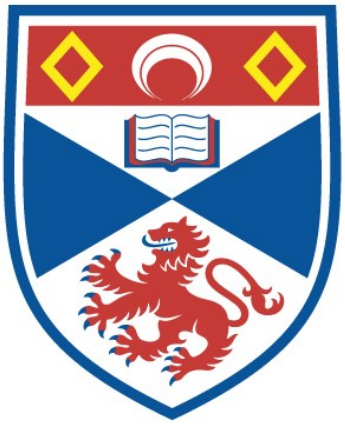
THE EFFECT OF HYPOXIA ACCLIMATION ON THE SKELETAL MUSCLE OF THREE FRESHWATER FISHES

Lynne Mills Bernard

A Thesis Submitted for the Degree of MPhil
at the
University of St Andrews



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THE EFFECTS OF HYPOXIA ACCLIMATION ON THE SKELETAL MUSCLE OF THREE FRESHWATER FISHES

A thesis submitted to the University of St. Andrews for the degree of Master of Science

by

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DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. I. A. Johnston.

CERTIFICATE

I hereby certify that Lynne M. Bernard has spent eight terms engaged in research work under my direction, and that she has fulfilled the conditions of the General Ordinance No. 12 (Resolution of the University Court No. 1,1967), and that she is qualified to submit the accompanying thesis for the Degree of Master of Science.

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SUMMARY

Many species of fish may be exposed to hypoxia in their natural habitats, either on a temporary or a more permanent basis. The survival of the animal may then be dependent on its ability to adapt to the conditions imposed upon it. There are several known ways in which a fish may adapt itself to coping with reduced oxygen levels such as by alterations in respiratory and ventilatory capacities and by changes in the oxygen carrying capacity of the blood, (Chapter 1). This study examines the effects of acclimation to hypoxia on the oxygen consumption, muscle ultrastructure and capillarisation of three species of freshwater fishes.

The species studied could all expect to experience hypoxia in their natural environments. The tench, Tinca tinca, would very probably become hypoxic during its winter hibernation buried in the mud at the bottom of ponds and rivers. The Crucian carp, Carassius carassius, inhabits sluggish or still waters where rotting vegetation reduces oxygen levels and it often spends its winters under complete or near complete anoxia in ice-locked ponds. The catfish, Clarias mossambicus, is found in tropical waters which dry out during certain seasons leaving the animals in hypoxic mud. (Chapter 2)

The effects of acclimation to hypoxia, ($P_{O_2} \sim 1.5$ KPa), for six weeks on the routine oxygen consumption of the tench and Crucian carp and of acclimation to hypoxia, ($P_{O_2} \sim 2.4$ KPa), for 27 days on the catfish have been studied (Chapter 3).

In the tench acclimated to aerated water, ($P_{O_2} \sim 17.6$ KPa), routine oxygen consumption was 32.7 mls O_2/Kg/h in aerated water. Acute exposure to hypoxia, ($P_{O_2} \sim 1.5$ KPa), led to a drop in oxygen consumption to 10.8 mls O_2/Kg/h, however after six weeks acclimation to hypoxia, oxygen consumption in hypoxic water had risen to

15.6 mls O_2/Kg/h, a 17% increase.

The routine oxygen consumption of the Crucian carp was measured during progressive hypoxia. In the carp acclimated to aerated water, routine oxygen consumption was 75.7 mls O_2/Kg/h in aerated water. During progressive hypoxia oxygen consumption fell in parallel with the decrease in water P_{O_2} to 16.2 mls O_2/Kg/h at $P_{O_2} \sim$ 1.5 KPa. After six weeks acclimation to hypoxia, ($P_{O_2} \sim$ 1.5 KPa), Crucian carp were able to maintain near constant oxygen consumption until water P_{O_2} fell to below 3.5 - 5.8 KPa. At $P_{O_2} \sim$ 1.5 KPa oxygen consumption was 34.0 mls O_2/Kg/h, significantly higher than fish acclimated to aerated water.

The catfish, Clarias mossambicus, is an air breathing species so oxygen consumption was measured in both the aquatic and aerial phases. Air-breathing frequency was 6.3/h in fish acclimated to aerated water, ($P_{O_2} \sim$ 15.3 KPa), and this was unaltered on acute exposure to hypoxia, ($P_{O_2} \sim$ 2.4 KPa), although aerial oxygen consumption was increased by 70%. This suggests that ventilation of the supra-branchial chambers is variable and therefore air breathing frequency is a poor measure of air breathing effort. Acclimation to hypoxia for 27 days increased air breathing frequency to 8.1/h. Total routine respiration rate for catfish acclimated to aerated water was 85.7 mls O_2/Kg/h. Of this 24.6% was attributable to air breathing. On acute exposure to hypoxia total routine respiration rate fell to 46.3 mls O_2/Kg/h. Acclimation to hypoxia for 27 days resulted in an increase in total routine respiration rate to 67.8 mls O_2/Kg/h largely as a result of a 164% increase in aquatic respiration from 10.4 to 27.5 mls O_2/Kg/h.

In all three species routine oxygen consumption was depressed by hypoxia and acclimation to hypoxia caused an increase in the capacity of the animals to extract oxygen from their hypoxic

environments (Chapter 3).

The myotomal fibre types of the tench, Crucian carp and catfish have been classified on the basis of histochemical staining for myofibrillar ATPase activity, succinic dehyrogenase activity and glycogen (Chapter 4). Three main fibre types are present classifiable as slow, fast aerobic and fast glycolytic. The patterns of innervation of the slow and fast glycolytic fibres have been examined by staining neuromuscular endplates and peripheral axons for acetylcholinesterase activity. All fibre types have a complex distributed innervation except the fast glycolytic fibres of the catfish which are innervated focally and terminally. This type of innervation is rare in teleosts.

A detailed examination of the ultrastructure of the slow and fast glycolytic fibres of tench acclimated to aerated and hypoxic water for six weeks has been made (Chapter 5). The major parameter affected was the mitochondrial volume fraction which decreased after acclimation to hypoxia in both the slow, (22.9 to 15.0%), and the fast glycolytic, (4.5 to 1.8%), myotomal muscle fibres, ($P < 0.01$). Intermyofibrillar mitochondrial populations, (4.4 to 1.2% slow; 0.6 to 0.04% fast), were affected to a greater extent than those in the subsarcolemmal zone, (18.5 to 13.8% slow; 3.9 to 1.8% fast). A significant increase in the myofibrillar volume fraction was observed after acclimation to hypoxia in both slow, (43.1 to 56.1%), and fast glycolytic fibres, (73.1 to 82.7%), ($P < 0.05$). Fibre cross-sectional area was unchanged after acclimation to hypoxia and actively growing fibres were observed in both groups.

The volume densities of mitochondria ($V_{v\ (mt,\ f)}$), capillary densities ($N_{A\ (c,\ f)}$) and various other measured and calculated indices of the structure and capillary supply have been determined. These parameters were determined for the slow and fast glycolytic myotomal muscles of all three species, acclimated to aerated and

hypoxic water, using low power electron micrographs (Chapter 6). They provide information on the structural limits relating to oxygen demand and supply in the swimming muscles.

In the tench capillary density ($N_{A\ (c,\ f)}$), was decreased after acclimation to hypoxia, falling from 2672 to 1371 in slow muscle and 676 to 250 in fast glycolytic muscle, decreases of 49 and 63% respectively.

In the Crucian carp mitochondrial volume density was significantly increased in both slow and fast glycolytic fibres after acclimation to hypoxia, (15% to 25% slow; 1.8% to 3% fast glycolytic). There was no significant change in the capillary density in either fibre type after acclimation to hypoxia.

The mitochondrial volume density and capillary density of the slow and fast glycolytic muscles of the catfish were not altered significantly by acclimation to hypoxia.

Thus, in terms of muscle ultrastructure and capillary supply, three very different responses to hypoxia acclimation have been found in the three species under study.

Considering only the parameters studied the following hypothesis are proposed to explain the changes observed.

Previous studies suggest that the increases in oxygen consumption observed after acclimation to hypoxia are due in part to increases in the oxygen affinity of the haemoglobin caused by decreases in the concentrations of the red cell nucleoside triphosphates.

The decreases in mitochondrial volume density and capillary supply in tench muscle after acclimation to hypoxia could be due to a reduced level of spontaneous locomotory activity. Tench experience

hypoxia mainly during their hibernation period where they remain totally quiescent. Energy requirement would be very low and metabolic rate would drop so some of the mitochondria and capillaries not required might be degraded.

The Crucian carp show a marked increase in volume density of mitochondria, especially in the slow muscles, after acclimation to hypoxia however capillary supply is little affected. This may represent an adaptation to increase the utilisation of circulating oxygen stores. The increased mitochondrial volume density would increase the extraction of oxygen from the blood thus decreasing the P_{O_2} of the venous blood which would facilitate an increase in the rate of oxygen transfer across the gills.

The mitochondrial volume densities and muscle capillary supply were unchanged in the air breathing catfish after acclimation to hypoxia. In this species it is likely that the increased ventilation of the suprabranchial respiritory organs together with the increased oxygen extraction at the gills obviated the need for significant changes in these parameters.

These studies have considered only a few of the possible adaptations which might occur in response to acclimation to hypoxia. There are many other factors such as the effects of the concentrations of the nucleoside triphosphates which would have to be studied before these hypothesescould be verified, thus there is much scope for further study on the subject of hypoxia acclimation in fish.

CHAPTER 1

INTRODUCTION

Terrestrial vertebrates tend to encounter variations in oxygen concentrations much less frequently than aquatic vertebrates. With the exception of high altitudes, the concentration of oxygen in air remains relatively constant, whereas that in natural waters often varies considerably due to factors such as temperature, degree of equilibrium with air, depth, photosynthesis, depletion by organisms, eutrophication, stratification and pollution. Environmental hypoxia may be temporary as happens during the night in some lakes due to plant respiration and lack of photosynthesis or permanent as in Lake Tanganika which is permanently stratified and therefore contains no oxygen below the thermocline (Coulter, 1967). Seasonal variations occur in most inland waters, caused by eutrophication, stratification and oxidation by iron (Reid, 1961) and sudden hypoxia may result from pollution of water by man's activities.

Due to the increased chances of aquatic vertebrates encountering reduced oxygen levels in their environment it seems probable that they will have retained the ability to adapt, be it on a temporary or a more permanent basis, to such reductions.

It has been shown that muscle is a relatively adaptable "organ". Changes in structure and metabolism have been shown to occur in response to variations in environmental temperature (Johnston and Maitland, 1980) and other factors such as starvation (Johnston, 1981a). Reductions in environmental oxygen levels ultimately lead to hypoxia at the cellular level therefore it seems likely that the effects of prolonged hypoxia would manifest themselves in the structure and metabolism of fish muscle.

Tissue oxygen availability, being the difference between the rates

of oxygen delivery by the blood and oxygen consumption by the tissues, varies greatly. It will be decreased not only under environmental hypoxia but also with increasing activity and temperature which thus become major factors determining tissue oxygen availability (Weber, 1982). The relationship between environmental and tissue oxygen availabilities is disordered by complex mutual interactions. Lowering the environmental oxygen levels of a fish will decrease the P_{O_2} gradient between the gills and the muscle mitochondria leading to lowered tissue oxygen levels (Weber, 1982). Although the animal may meet the problem of lowered oxygen availability with evasive action this is often not possible and so it must adapt physiologically if it is to survive.

Fish Locomotory Muscle

On average 40 - 60% of the fish body is composed of muscle. Fish muscle is particularly suitable for physiological study since it is easily dissected into homogenous populations of single fibre types. This chapter will deal with a general review of fish muscle followed by a short account of anaerobiosis in fish.

The structure and fuction of fish locomotory muscle

Gross Anatomy

There are over 25000 species of fish each one adapted to its own particular mode of life. The range of body forms and modes of locomotion is wide and beyond the scope of this study but generally swimming is achieved through contractions of the myotomal muscalature resulting in undulations of the body and, by use of the paired and unpaired fins. In most species the myotomal muscle provides the main propulsive force therefore this review will concentrate mainly on studies of myotomal muscle.

The lateral body muscalature is divided into a series of segments termed myotomes. The shape of the myotomes varies from a V shape in Amphioxus through a shallow W shape in the Agnatha and increasing phylogenetically to a deep W in the teleosts (Nursall, 1956; Alexander, 1969). The myotomes are divided by connective tissue myosepta into which the muscle fibres insert via small tendons.

The bulk of the body muscalature consists of fast or white muscle whose fibres are arranged in a complex pattern within the myotomes. In general the superficial white fibres run parallel to the long axis of the body but with increasing depth they make angles of up to 40° with the vertebral column. These orientations combine through successive myotomes to describe a series of helices with the axes running parallel to the long axis of the body (Alexander, 1969). Patterns of orientation of the white fibres vary from one phylogenetic group to another and even in different parts of the same fish. For example the pattern found in the teleost body is very different from that of the selachians, however in the caudal region of the teleosts the pattern reverts to that of the selachians (Alexander, 1969). Functionally it is thought that this arrangement of fibres allows a similar degree of sarcomere shortening at different body flexures (Alexander, 1969; Bone, 1978a), thus maximizing power output.

The other main fibre type, the slow or red fibres are situated under the skin and generally run parallel to the longitudenal axis of the body.

Muscle Fibre Types

Historically, muscle fibres in fish have been classified according to their colour. As early as 1678, Lorenzini had described red and

white fibres in the muscalature of Torpedo. This method of classification has been retained by workers studying fish muscle as the simplest although rather unsatisfactory method (Johnston, 1981a) and the terms red and white will be used in this review except where data is available to classify fibres according to their physiological properties.

Bárány (1967) has demonstrated that myofibrillar ATPase activities parallel the unloaded speeds of shortening in a wide range of vertebrate muscles, in general the white fibres having a much higher activity and speed of shortening than red fibres. These properties give a further method of classifying muscle fibres. In many cases the terms 'slow' and 'fast' are used synonymously with 'red' and white however there are many technical difficulties involved in measuring speeds of shortening in fish muscle so few values are known and exceptions have been found such as the fast red fibres in bat incthothyroid muscle (Revel, 1967).

The number of fibre types distinguishable by their differing histochemical, biochemical, ultrastructural and physiological properties found in any species ranges from two to five and their presence or absence and location in the body depends on species and group but basically there are always two main fibre types present, the red and the white. The structure and function of these fibres has been reviewed several times, most recently by Bone (1978a) and Johnston (1981a).

Despite great variations in the body morphology of the fishes the distribution of the fibre types within the body muscalature remains fundamentally the same.

The red muscle is situated just under the skin in a superficial layer. It may encircle the whole body as in the dogfish, Scyliorhinus canicula or form a wedge under the lateral line as in most of the teleosts. It has been shown that the proportion of red muscle in the

myotome is related to the swimming behaviour of the fish. Greer-Walker and Pull (1975) have examined 84 species and their evidence shows conclusively that fish with high cruising speeds have more red muscle than those with slower swimming speeds. Red muscle fibres constitute less than 10% of the total myotomal muscalature in most fish, however they may comprise as much as 48% body weight as in Sardinia melanosticta (Fujikawa & Naganuma, 1936). Proportions of red muscle vary considerably, not only between species but along the trunk and with increasing body size (Magnuson, 1973). Some members of the family Scombridae have an internally located mass of red muscle separate from the superficial layer. This muscle is physiologically different from the superficial layer and is associated with a vascular counter-current heat exchange system used to maintain elevated brain and slow muscle temperatures (Sharp & Pirages, 1978).

The white muscle lies beneath the red and constitutes the bulk of the myotomal muscle mass.

Most fibres fall into one of three main groups classified according to colour as red, pink or white fibres. The small percentage remaining form minor groups whose functions are as yet poorly understood.

Histology, Histochemistry and Biochemistry

Red Fibres

In general red fibres are characterised by several factors. High concentrations of myoglobin and numerous capillaries give rise to the red colour (Matsuvra & Hashimoto, 1954). Myoglobin greatly facilitates the diffusion of oxygen in muscle tissue (Hemmingsen, 1965).

Biochemical determinations and histochemical studies have shown low activity and staining for Ca^{2+} activated myofibrillar ATPase (Nag, 1972; Johnston et al., 1974; Bone & Chubb, 1978; Bone & Johnston,

1982). This is in keeping with the positive correlation between biochemical measurements of myofibrillar ATPase activity and contraction velocity (Bárány, 1967).

Average fibre diameter is small compared to white fibres and the tissue is well vascularised (Mosse, 1978). Capillary density is related to aerobic capacity and has been shown to be significantly higher in red than in white muscle in most species (Mosse, 1978), though certain pelagic species such as yellow tail scad, pilchard and mackeral have a more highly developed capillarization of the white muscles (Mosse, 1979).

Enzymatic studies have shown the red fibres to be primarily dependent on aerobic metabolism for their energy supply. Gordon (1972) has measured oxygen consumption of the isolated red muscle of ten species of marine teleost and has shown it to be much higher than that of white muscle. Activities of the various enzymes involved in aerobic metabolism eg hexokinase, malate dehydrogenase, succinate dehydrogenase and cytochrome oxidase are high and histochemical staining for the tricarboxylic acid cycle enzymes is intense (Johnston, Davison & Goldspink, 1977).

Naturally exceptions to these generalisations occur. For example the icefish, Champsocephalus gunnari, is an interesting exception to the correlation between muscle pigmentation and aerobic capacity. Although mitochondria occupy 45% of the volume of the slow fibres in this species the muscle colour is white due to the absence of myoglobin (Walesby, Nicol & Johnston, 1980).

Pink Fibres

Pink fibres have been shown to be present in many, but by no means all species of fish. They are situated between the red and white

muscle layers and are sometimes referred to as intermediate fibres due to their intermediate colour and biochemical properties.

Biochemical studies show that the pink fibres have aerobic enzyme and myofibrillar ATPase activities between those of the red and white fibres. Histochemically they have an alkaline stable (pH 10.4) myofibrillar ATPase activity (Johnston et al., 1974; Ward & Goldspink, 1975). In species such as the carp, Carassius carassius and the mirror carp, Cyprinnus carpio the pink fibres account for approximately 10% of the trunk muscalature, proportionally more than the red fibres (Johnston et al., 1974). The composition of the myosin light chains in vertebrates have been shown to be characteristic of fibre type (Lowey & Risby, 1971). In carp, red fibres have two species of myosin light chain whereas pink and white fibres both have three, characteristic of fast muscle myosins (Focant, Huriaux & Johnston, 1976; Johnston, Davison et al., 1977).

White Fibres

Typically white fibres have low concentrations of myoglobin and a poor vascular supply giving rise to the white colour of the tissue. They form a heterogenous group with respect to fibre size and usually have a greater mean diameter than red fibres. In trout there is a continuous distribution of fibre diameter between 15 and 90 μm in white fibres compared to 5 - 40 μm in red (Johnston, Ward & Goldspink, 1975). This range of fibre size in white muscle may represent different stages in growth rather than distinct fibre types (Johnston, Ward et al., 1975; Korneliussen, Dahl & Paulsen, 1978; Johnston & Moon, 1980 b) as it has been shown that, in contrast to most other vertebrates, fibre number continues to increase throughout life in fish (Greer - Walker, 1970).

White fibres have been shown to be primarily dependent on anaerobic glycogenolysis for their energy supply. Gordon (1972) has measured oxygen uptake in vitro of the white muscle of 39 species of marine teleost and has shown it to be between two and twelve times less than that of red muscle.

Activities of the enzymes involved in aerobic metabolism are low and Mg^{2+} and Ca^{2+} activated myofibrillar ATPase activity is around three times that of red (Johnston, Frearson & Goldspink, 1972; Nag, 1972; Johnston & Tota, 1974). Histochemical staining for SDHase is negative whereas that of ATPase is intense. There have been relatively few studies of capillary supply to fish muscle fibres but for most species capillary density is much lower in white muscle (Egginton, 1982).

Minor Fibre Groups

Several minor fibre groups and subgroup divisions of the three main fibre types have been described. Bone (1978a) has described a single fibre layer of large diameter SDHase and Ca^{2+} activated ATPase negative fibres immediately below the skin in the dogfish, Scyliorhinus canicula, the function of which is unclear. Bone has also divided the red and white muscles in the dogfish into two subgroups on the basis of slightly differing histochemical, biochemical and physiological properties. The outer red fibres have higher SDHase, lower myofibrillar ATPase and smaller diameters than the red fibres adjacent to the white muscle. The outer layer of white fibres have a higher proportion of mitochondria, more abundant capillary supply and a slightly higher myofibrillar ATPase activity than the deep white layers.

The internalised red muscle mass in the tuna, Katsuwonus pelamis, has been shown to differ from the superficial red muscle. The internalised muscle has a smaller mean fibre size and a significantly

higher staining for SDHase activity (Bone, 1978).

Small diameter fibres with positive SDHase activity and low myofibrillar ATPase activity have been found scattered among the fast fibres giving a mosaic type arrangement in several species including the cod, Gadus morhua, (Greer-Walker, 1970), the rainbow trout, Salmo gairdneri, (Johnston et al., 1975), and Lophius (Bone & Chubb, 1978). These probably represent stages in growth rather than distinct fibre types (Johnston et al., 1975; Korneliussen et al., 1978; Johnston & Moon, 1980 b).

Ultrastructure

Studies of the ultrastructure of muscle fibres provide a more complete functional picture of the various fibre types. Quantitative studies of fish myotomal muscle have been made by Patterson & Goldspink, 1972, 1973 (goldfish and crucian carp) Kryvi, 1977, (Entmopterus spinax and Galeus melastomus) Kryvi & Totland, 1978 (Chimaera monstrosa) Bone, 1978b,(Tuna - Scomber and Katsowonus) Walesby & Johnston, 1980 (Notothenia rosii) Johnston & Maitland, 1980 (crucian carp) Johnston & Bernard, 1982a,b(tench). These studies show similar ultrastructural characteristics for homogenous fibre types between species.

The most noticeable difference between red and white fibres is in the percentage fibre volume occupied by mitochondria. Red muscle shows a very high mitochondrial density, the fractional volume lying between 25 and 35% (Kryvi, 1977; Bone, 1978a; Johnston & Maitland, 1980; Kryvi, Flood & Guljaer, 1980; Walesby & Johnston, 1980; Johnston, 1981b; Patterson & Goldspink, 1982). This is almost as high as that of active mammalian muscles such as mouse ventricle (Bossun, Sommer & Waugh, 1978).

Pink fibres have intermediate fractional volumes of mitochondria and white fibres very low amounts. Mitochondria in the red fibres are

generally located both subsarcolemmally and intermyofibrilally, the majority being subsarcolemmal, whereas in the white fibres there are only scattered subsarcolemmal mitochondria, the majority of the small percentage present being located between the myofibrils (Kryvi et al., 1980).

The percentage volume of fibres occupied by myofibrils increases in order red< pink < white, rising from 40 - 60% in red muscles to 80 - 96% in white. An interesting characteristic apparently unique to teleosts lies in the structure of the myofibrils. In transverse section the periferal myofibrils are elongate and ribbon like, radiating outwards and resembling the spokes of a wheel in small fibres.

Comparisons with amphibian and mammalian muscle fibres have shown that both the T-system and the sarcoplasmic reticulum are much more highly developed in fish (Johnston, 1980a). The sarco-tubular system occupies a greater fractional volume in the white that the red fibres (Kryvi, 1977; Nag, 1972; Akster, 1981). This would be expected due to the requirement for an increase in relaxation rates from red to white. In mammalian muscles the T-tubules are situated at the A - I boundary, however studies indicate that fish myotomal muscle differs in this respect by having the T-tubules situated at the level of the Z-disc (Franzini, Armstrong & Porter, 1964; Kilarski, 1966; Nag, 1972; Patterson & Goldspink, 1972; Kryvi, 1977). Page (1968) has shown that generally fibres with shorter actin filaments have the T-system located at the Z-line while fibres with longer actin filaments have the T-system at the A - I junction. Akster (1981) proposes that the position of the T-system and adjacent sarcoplasmic reticulum at the A - I junction in fibres with long actin filaments may be a structural adaptation to ensure a sufficient calcium supply to all troponin/tropomyosin complexes during contraction.

The M-line is prominent in fish muscle and the Z-line is thicker in white than in red fibres (Patterson & Goldspink, 1972).

Stored supplies of the fuels required for energy production vary between muscle types. Glycogen is stored in the form of small particles or rossettes mainly in the red fibres. though exceptions are found such as in the tuna, Euthynnus pelamis, which has higher glycogen stores in the white fibres (Guppy et al., 1979). Glycogen stores vary considerably both within and between species depending on nutritional and physical status (Love, 1970). Lipid is stored either as droplets within and between the fibres or as a superficial layer under the skin as in the anchovy (Johnston, 1980). There is generally more fat present in and around the red fibres.

Innervation

In all fish species the red fibres are innervated in the same way, multineuronally, by small diameter, myelinated axons, terminating in 'en grappe' type end plates (Barets, 1961; Bone, 1966, 1970; Best & Bone, 1973). Bone has demonstrated that in the elasmobranchs and teleosts there are at least two axons innervating each red fibre. Subjunctional folds are present under the terminations in elasmobranchs (Bone, 1972), but not in teleosts (Nishihara, 1967). and acetylcholinesterase has been demonstrated to be the main transmitter present in all slow fibres (Bone, 1978a).

The innervation of the white fibres varies depending on the phylogenetic group. This is of interest as it implies that the pattern of innervation may serve as a taxonomic character in classifying fish. In the elasmobranchs and primitive teleosts the white fibres are focally innervated usually at one myoseptal end (Bone, 1978a). In the dogfish each muscle fibre is innervated by two separate axons which fuse together

to give a single end plate (Bone, 1964, 1972). Bone and Ono (1982) have studied the innervation pattern of over 230 teleost species and have found the terminally innervated pattern present in the basal teleost groups with a trend towards distributed innervation in the Neoteleostei. Stomiiforms possess a rather different distributed pattern which it is suggested is the early transitional stage from terminal to distributed innervation patterns. There appears to be a distinct functional difference in the distributed and terminal innervation patterns (Bone & Ono, 1982).

The multiple, distributed innervation of the advanced teleosts consists of a diffuse network of nerves fanning out over the fibres from branches of the spinal nerves which run into the myosepta. Each fibre thus receives branches from numerous axons (Barets, 1961; Bone, 1964). Subjunctional folds are absent, the nerve terminals being buried in the sarcolemma of the fibres (Nishihara, 1967). Again only acetylcholinesterase has been found at the junctions, although two types of endplate terminal vesicle have been demonstrated to occur in the elasmobranchs (Bone, 1972). One type is the typical 50 nm diameter whilst the other is a larger dense core vesicle up to 100 nm diameter.

Figure 1 shows an illustration of the distinctive features, generally found in the three main fibre types, discussed so far.

Electrophysiological and Mechanical Properties

Electrophysiologically the red fibres of fish resemble the true slow fibres of the amphibia. Depolarising pulses elicit junction potentials which activate the fibres (Hagiwara & Takahashi, 1967; Stanfield, 1972). Stanfield has produced some evidence to suggest that

the slow fibres may be capable of generating action potentials.

There are few studies on the mechanical properties of fish muscle. Fish red fibres are very small and their attachment to equipment technically difficult due to their myoseptal insertion. Bone & Johnston (1978) have studies the properties of small fibre bundles from the dogfish myotome. Red fibres respond to a supramaximal stimulus with a slow contraction ($t_{\frac{1}{2}}$ = 100 ms). Fused tetani are produced at frequencies above 8 Hz and twitch-tetanus ratios of around .0 . 5 occur at 10 Hz. The response of red fibres from the adductor operculi muscles of the teleost, Tilapia mossambica, are very different to those of the dogfish. They only respond to stimulation frequencies in excess of 5 - 10 Hz and produce graded fused tetani, reaching a maximum at 250 - 300 Hz (Flitney & Johnston, 1979).

The electrophysiological properties of the white fibres differ from those of the red and also between focally and multiply innervated groups. The response of the focally innervated fibres is similar to that of amphibian fast fibres, depolarisation giving rise to a large overshoot in spike potential and propagated action potentials (Hagiwara & Takahashi, 1967).

In contrast, the few studies on multiply innervated fast fibres of teleosts have shown that stimulation of the spinal nerves elicits two kinds of response, either an action potential resulting in a fast twitch or junction potentials leading to a graded local contraction of the muscle (Hudson, 1969). Hudson found that in some preparations it was not possible to elicit action potentials whilst in others a single junction potential gave rise to an action potential. Such conflicting results suggest that more work is necessary on the electrophysiological properties of multiply innervated muscle before positive statements

about the 'in vivo' action can be made.

The mechanical properties of focally and multiply innervated fibres also differs. The mechanical response of focally innervated dogfish white fibres to a single supramaximal stimulus is a fast twitch ($T_{\frac{1}{2}} \approx 20$ ms). Twitch-tetanus ratios of around 0.5 occur at 10 Hz (Johnston, 1981a). In the multiply innervated white muscle from the adductor operculi muscles of Tilapia mossambica graded, fused tetani were produced at stimulation frequencies above 5 - 10 Hz and reaching a maximum at 250 - 300 Hz (Flitney & Johnston, 1979). The rate of tension development was found to be critically dependent on stimulation frequency and at 200 Hz was 6.5 times greater in fast than slow fibres. Johnston (1980) has found a similar dependence of the rate of tension development on stimulation frequency in the polyneuronally innervated fast fibres of the cod, in a comparison study with the focally innervated fast fibres of the cuckoo ray, Raija naevus. Tetanic fusion frequencies and maximal tension frequencies were substantially higher in the polyneuronally innervated cod fibres.

The order in which the fibre types are recruited during swimming has been the subject of few studies. Evidence from the few species which have been studied suggests that the type of fast muscle innervation governs the pattern of fibre recruitment. In the focally innervated fast fibre species the division of labour is well defined. In the dogfish (Bone, 1966) and the Pacific herring (Bone et al., 1978) electromyographical measurements from the muscles show that only the slow fibres are active at low, sustainable cruising speeds. At higher burst speeds the fast fibres are recruited and the fish fatigues rapidly. For example the Pacific herring can sustain speed of $>$ 4 lengths/s for several hours and only the red fibres are recruited

however at speeds $\geq$ 5 lengths/s white fibres are recruited and the fish fatigues in 1-2 minutes (Bone et al., 1978).

Fibre recruitment in seven species of teleost with polyneuronally innervated fibres have been studied (skipjack tuna - Rayner & Keenan, 1967; Brill & Dizon, 1979; rainbow trout - Hudson, 1973; carp - Johnston et al., 1977; striped bass and bluefish - Freadman, 1979; brook trout - Johnston & Moon, 1980b; coalfish - Johnston & Moon, 1980a). In these fish, at low speeds only the slow fibres are active, however as swimming speed increases, a threshold is reached where fast fibres are recruited. These speeds vary between species eg 0.8 - 1.9 lengths/s in coalfish (Johnston & Moon, 1980a) and 4 - 5 lengths/s in bluefish (Freadman, 1979) and can be maintained indefinitely by the fish. Interestingly e.m.g. activity from the fast muscle at high cruising speeds is similar to that of the slow muscle whereas at very high burst speeds higher amplitude spike potentials are seen.

The recruitment of the intermediate pink fibres has been studied in only one species, the carp, by Johnston et al. (1977). The pink fibres were recruited before the white and fibre recruitment was in the order red > pink > white with increasing speed. It appears that recruitment is also dependent on depth within the myotome, the superficial white fibres being recruited before the deeper white (Johnston & Moon, 1980b).

Control of contraction in fish muscle

Like all vertebrate muscles the contraction of fish muscle is controlled by nervous impulses stimulating the release of calcium ions from the sarcoplasmic reticulum. The calcium binds to troponin C and activation of the myosin crossbridges occurs giving rise to contraction.

An interesting feature of fish muscle is the presence of high concentration of the cytoplasmic calcium binding proteins - parvalbumins.

These are found in all vertebrate fast muscles but are present in particularly high concentrations (≈ 15% soluble proteins) in fish fast fibres (Le Peuch, Demaille & Pechère, 1978).

Parvalumins show a high degree of similarity to troponin C. the myosin P-light chain and calmodulin (Collins, 1976; Perry, 1979). They are able to inhibit myofibrillar ATPase activity by binding Ca^{2+} (Pechère et al., 1977) and the bound Ca^{2+} may be exchanged and accumulated by the S.R. vesicles (Gerday & Gillis, 1976).

It seems likely that the function of parvalbumins is to induce rapid relaxation in white fibres. During contraction only a small amount of the calcium released from the S.R. will initiate contraction. the majority of the ions being quickly sequestered by the parvalbumins. This means only a transient activation of the contractile apparatus will occur. This very short activation/relaxation cycle would allow a very high tailbeat frequency to be achieved during burst swimming (Gerday & Gillis, 1976; Pechère et al., 1977).

Parvalbumins in fish and vertebrate muscles have recently been reviewed by Gerday (1982).

Metabolism

Metabolism is such an immense subject that it is impossible to review it adequately within the realms of this thesis. Excellent accounts of intermediary metabolism in fishes are given by Hochachka (1969), Tarr (1972) and Driedzic and Hochachka (1978). so a short review of muscle metabolism in fishes only is given.

Like all animals fish depend on the splitting of ATP to provide the energy to power muscle contraction, thus muscle metabolism is geared towards the production and utilisation of this molecule. The metabolic cost of swimming increases approximately exponentially with speed,

due to the hydrodynamic drag forces imposed on fish at speed. Oxygen uptake also increases exponentially with speed, rate being dependent on body size and environmental factors (Brett, 1972) until the aerobic threshold is reached after which rate of uptake cannot increase any further. Although fish use less energy for locomotion than mammals, their scope for aerobic activity is up to 100 times less (Brett, 1972) thus they show a high dependence on anaerobic metabolism. Figure 1 : 2 gives a simplified outline of the pathways involved in ATP production.

In vitro enzyme determinations under optimal conditions of the key enzymes involved in energy metabolism give an indication of the metabolic capacities and importance of the various pathways in different muscle fibre types. The separation of the different fibre types into discrete regions in fish muscle facilitates their excision for enzymatic studies. Several species have been studied including the eel (Boström & Johansson, 1972), dogfish (Crabtree & Newsholme, 1972), rainbow trout (Crabtree & Newsholme, 1972; Johnston, 1977), mirror carp (Johnston et al., 1977). tuna (Guppy, Hulbert & Hochachka, 1979) and an Antarctic teleost (Walesby & Johnston, 1979).

Generally the white muscles show activities of glycolytic enzymes such as phosphorylate and phosphofructokinase to be 2 - 3 times greater than in red muscle whereas markers for oxidative pathways such as citrate synthetase have higher activities in red muscle. Concentrations of the metabolites ATP, ADP, AMP, free phosphate and phosphoryl creatine tend to be higher in white than red muscle which would be expected as ATP turnover has been shown to be three times greater in white muscle compared to red. Activities of the ATP producing enzymes, creatine kinase and adenylate kinase parallel these differences (Johnston & Moon, 1980). However, this pattern of enzyme activities and metabolite

concentrations does not hold for all species. Boström and Johansson (1972) have shown that for the eel, Anguilla anguilla, in its silver stage, glycolytic pathway enzymes have equal activities in both red and white muscles.

Enzyme activity levels and metabolite concentrations are also affected by such factors as developmental stage (Boström & Johansson, 1972), starvation (Moon & Johnston, 1980) temperature acclimation (Hazel & Prosser, 1974) and exercise training (Johnston & Moon, 1980).

The two main fuels for ATP production in fish are lipids and glycogen. In elasmobranchs lipids are stored in the form of triacyl glycerol in the liver and activities of the enzymes of ketone body oxidation, eg 3-hydroxy-butyrate dehydrogenase, are high, especially in the red muscles (Zammit & Newsholme, 1979). In contrast, teleosts often have lipid stored in the viscera and dispersed between the muscle fibres as well as in the liver. Activities of the enzymes of fatty acid oxidation, carnitine palmitoyl transferase and triacyl glycerol lipase, are high and high concentrations of non-esterified fatty acids are found in the plasma (Zammit & Newsholme, 1979). Lipids are utilized to a much greater extent by slow muscles in both teleosts and elasmobranchs (Jonas & Bilinski, 1964; Crabtree & Newsholme, 1972; Bilinski, 1974).

Glycogen is stored in the liver and in muscle fibres and is utilised by both red and white fibres as a fuel source during steady swimming (Pritchard et al., 1971; Johnston & Goldspink, 1973b). It appears to be used mainly aerobically by the red muscles and anaerobically by the white resulting in the production of lactate. In the crucian carp, the red muscle utilizes glycogen at two to three times the rate of white muscle at a constant swimming speed of 3 lengths/s (Johnston & Goldspink, 1973a). However, only 15 - 20% of the total glycogen utilized can be

accounted for by the red muscle since they comprise only 7% of the trunk. The rest must be metabolised by the white muscle.

Metabolism in fish during swimming is poorly understood due to the difficulties involved in obtaining proper controls, however some general conclusions can be drawn. Oxygen uptake can increase by a factor of 10 - 15 times (Bennet, 1978) and the highly aerobic red muscle almost certainly receives a significant proportion of the cardiac output during steady swimming. The importance of aerobic glycolysis varies considerably between species for both red and white muscle. A good correlation between hexokinase activities and maximum capabilities for glucose oxidation has been demonstrated by Crabtree and Newsholme (1972). In two species of trout and the skipjack tuna hexokinase activities in the white muscle are high and comparable to those of the red muscle (Guppy et al., 1979; Johnston & Moon, 1980). This suggests a significant aerobic capacity in the white muscles of these species. Supported by other evidence such as high mitochondrial numbers (Nag, 1972) and high activities of citrate synthetase and cytochrome oxidase in trout white muscle (Johnston & Moon, 1980) it seems likely that in these species the aerobic capacity of the white muscle is probably sufficient to support sustained activity.

This aerobic capacity of the white muscle does however seem to be the exception rather than the rule. In most species it appears that anaerobic pathways are the most important means of providing energy for ATP production during sustained activity. On the basis of oxygen consumption measurements and efficiency of swimming, Smit et al. (1972) have calculated that for the goldfish, (Carassius auratus), as much as 80% of their energy requirements are provided by anaerobic pathways.

Lactate is the major end product of anaerobic metabolism during

steady or burst swimming. Most of this is probably oxidised to pyruvate in the gills, liver, red muscle and kidneys (Bilinski & Jonas, 1972; Bilinski, 1974). In trout burst swimming results in a 50% depletion in muscle glycogen in 15 sec. (Stevens & Black, 1966), almost all of which is converted to lactate (Wardle, 1978). Lactate levels return to normal in 30 - 60 min in red muscle but may take up to 18 h in white muscle (Black et al., 1961; Johnston & Goldspink, 1973 a,b,c.).

Hypoxia, Anoxia and Anaerobiosis

There are several ways in which a fish may become hypoxic, eg severe muscular activity, lowered environmental oxygen levels and blocking of oxygen diffusion at respiritory surfaces, but essentially the end result is an insufficiency of oxygen at the cellular level. When the supply of oxygen available to an animal is not sufficient to meet its needs it must either reduce its overall demand or respire anaerobically. In the fishes most of the energy for activity of the white muscles is derived anaerobically so obviously the potential for sustained anaerobiosis during environmental hypoxia is present. This section will concentrate only on the responses and adaptations of fish to hypoxia and anoxia.

It has long been known that many species of fish have impressive capacities for tolerating hypoxic or anoxic conditions. The Indian cyprinid, Rasbora daniconius (Ham.) can survive in a sealed container for more than 100 days (Mather, 1967), and the crucian carp, Carassius carassius, can survive for 5½ months in ice locked ponds under strictly anaerobic conditions (Blažka, 1958). The importance of this anaerobic tolerance is obvious - the survival of the species will be highly dependant on the ability to survive environments with low oxygen

levels if and when they should be encountered.

Most fish fall into the category of metabolic regulators. On exposure to falling oxygen levels they will maintain a constant oxygen consumption down to some level of P_{O_2} referred to as the critical pressure (P_c) after which oxygen consumption falls rapidly. This is dependant on species and temperature (Prosser, 1973) and does not necessarily relate to the fishes natural environment. Marvin and Heath (1968) have shown that although the brown bullhead catfish often encounters hypoxic water in its natural environment, it nevertheless has a higher P_c than the trout, a fish which seldom encounters any reduction in its natural water oxygen levels.

Another important factor which is very species variable is oxygen uptake capacity. Large differences exist between species with respect to uptake capacities so that a particular oxygen level proving hypoxic to one species will not necessarily be hypoxic to another (Saunders, 1962; Beamish, 1964; Jones et al, 1970).

An important factor in determining a fishes ability to withstand hypoxia is its ability to build up an oxygen debt. All animals have a certain capacity to use energy without oxygen consumption through the utilization of existing energy stores such as ATP and creatine-phosphate and by some anaerobic energy producing metabolic pathways of which glycolysis is by far the most important (Van den Thilart, 1976, 1977). Utilization of anaerobic energy will not only strongly influence performance efficiency but will also increase the oxygen consumption during the recovery period. The extent to which an oxygen debt is built up during hypoxia or anoxia gives some indication of the efficiency of the fishes anaerobic metabolism. Those building up no or very small oxygen debts tend to have high anaerobic capacities. Blažka (1958) found no significant increase in oxygen consumption in the crucian carp after a period of anoxia, however he did observe a

significant increase in the trout. Marvin and Burton (1973) demonstrated a remarkable oxygen debt in trout, a small one in bluegills and no oxygen debt at all in catfish.

The initial responses of fish on encountering hypoxia are generally threefold (a) Hyperventilation resulting from an increase in the rate of opercular movement and/or the volume of water drawn over the gills at each stroke causes an increase in the total amount of water in contact with the respiritory surfaces (Saunders, 1962; Holeton & Randall, 1967; Watters & Smith, 1973; Lomholt & Johansen, 1978).

(b) Either bradycardia or tachycardia: Both effects have been demonstrated and would theoretically increase the amount of oxygen taken up by the blood. Decreased heart rate would cause the blood to flow more slowly through the gills thus increasing the time for oxygen loading to occur. Increased heart rate would increase the amount of blood passing over the respiritory surfaces. Cardiac stroke volume may also alter. Hypoxia raises total cardiac output in winter flounder and carp (Cech et al., 1977; Itazawa & Takeda, 1978), but in the trout increased stroke volume is offset by a decreased heart rate. Hughes and Umezawa (1968) have shown for the trout, carp and goldfish that arterial oxygen level increases as the ratio of ventilation volume to cardiac output increases.

(c) Redistribution of cardiac output to only the most essential organs conserves both oxygen and energy (Hochachka, 1980).

These adjustments are however only effective until the increased energy cost of ventilating the gills outweighs the benefits from increased oxygen availability. At this point ventilation rate drops to very low values and the animal must become dependant on anaerobiosis for its energy supply.

The responses described so far are only immediate responses. After a time period of exposure to hypoxia adaptation occurs and more

permanent physiological changes may occur. The effects of long term acclimation to hypoxia have been studied by only a few authors (Beamish, 1964; Kutty, 1968b; Van den Thillart, 1977; Lomholt & Johansen, 1978; Weber & Wood, 1978; Weber at al, 1978).

Wood and Johansen, studying the eel, Anguilla anguilla, show three stages in adaptation to hypoxia. Initially breathing rate increased. After two days this could not be maintained and lactic acidemia, indicating oxygen debt, occurred. By the seventh day the blood had acquired an increased oxygen affinity and capacity so more oxygen can be delivered to the tissues and normal acid-base status is restored.

Studies by Beamish(1964) and Kutty (1968b) showed remarkable differences in oxygen dependancy in hypoxia acclimated goldfish, Carassius auratus, however Van den Thillart's (1977) work did not confirm these results. Kutty observed a significant decrease in oxygen consumption below 50% air saturation whereas when measured at night, Van den Thillart did not find any decrease until below 10%. Differences in oxygen consumption rates between hypoxia and normoxia acclimated goldfish disappeared at night. Reduction of activity seems to be a primary effect of hypoxia acclimation. Maximal routine rates of oxygen consumption were markedly depressed below 10% air saturation and reached minimum levels around 4% air saturation in goldfish. Spontaneous activity normally occurring during the day was strongly depressed (Van den Thillart, 1977). Hypoxia acclimation lowers the P_c and increases the survival time at sublethal oxygen tensions (Van den Thillart, 1977). Lomholt and Johansen (1978) working with the carp, Cyprinus carpio, and using a more sophisticated measuring technique showed higher values of V_{O_2} for the normoxia acclimated group correlating with a higher spontaneous activity level when compared at the conditions of acclimation. When both groups were

compared in hypoxic water (P_{O_2} 10 - 30 mm Hg) the V_{O_2} of the hypoxic acclimated fish was 30 - 40% higher. Ventilation of the gills in hypoxic water was much less in the hypoxic acclimated fish and oxygen extraction was higher.

Of major importance in the acclimation of fish to hypoxia is the effect of the concentrations of the nucleoside triphosphates on the affinity of the haemoglobin for oxygen. Adaptation to low oxygen levels results in a marked fall in the concentrations of these compounds, allowing greater oxygen uptake by the red cells. Adenosine triphosphate and guanosine triphosphate act in this way, GTP being the most important due to its greater effect on oxygen affinity and larger range of possible concentrations within the cell (Weber, Lykkeboe & Johansen, 1975, 1976; Weber & Wood, 1978; Weber et al., 1978; Weber, 1982). Weber and Lykkeboe (1978) have shown for the carp, Cyprinius carpio, that concentrations of the nucleoside triphosphates fall from 9.05 to 4.98 mM/ℓ cellsafter one month's acclimation to hypoxia. The concentration of ATP fell from 5.74 to 4.14 mM/ℓ cells and that of GTP, by a greater amount, from 3.71 to 1.54 mM/ℓ cells. Similar effects have been demonstrated for other species and it seems that the concentration of ATP directly reflects the oxygen concentration to which a fish has become habituated (Weber, Lykkeboe & Johansen, 1975, 1976; Weber & Wood, 1978; Weber et al., 1978; Weber, 1982).

As yet it is not clear how lowered nucleoside triphosphate concentrations reduce the blood oxygen affinity but it is thought that the effect may be directly through reduced allosteric interaction and indirectly through a decreased erythrocyte pH resulting from an altered Donnan distribution of protons across the red cell membrane (Wood & Johansen, 1973; Weber & Wood, 1974). The main influence on the capacity of the blood for carrying oxygen seems to be through the effect of these modulating systems. There have been increases in the

abundance of blood cells, haematocrit and haemoglobin concentrations shown for some species, however other species show little or no change in these characteristics following acclimation to hypoxia. An increased haematocrit does not necessarily mean an increased haemoglobin concentration. Soivo et al. (1974) showed that in salmonid species asphyxia causes the red cells to swell, so increasing the haematocrit value. Salmo gairdneri increases urine flow thus concentrating the blood and increasing haemotocrit (Swift & Lloyd, 1974).

Little work has been done on the effect of hypoxia on the concentrations of metabolites and activities of metabolic enzymes.

It appears that during hypoxia glycogen serves as the primary energy source (Hochachka, 1972). Kutty (1972) suggests that protein catabolism also increases. Acclimation to hypoxia appeared to cause only slight shifts in the enzyme activity patterns of goldfish (Van den Thillart, 1977). At low temperatures hypoxia caused decreases in GPT activity whereas at higher temperatures succinate oxidase activity was increased. Johnston and Bernard (1982b) have shown enzyme activities consistent with an enhanced capacity for glycolysis in the red muscle and liver of the tench.

Obviously it is important that the enzymes of the glycolytic cycle should be able to function actively under anoxic conditions and should not be affected by end product build-ups such as lactate. Johnston (1975b) found that the pH optimum of pyruvate kinase in Carassius carassius was broad and low, and so,well adapted to perform during sustained anaerobiosis.

During normal aerobic metabolism energy is produced by the oxidation of reduced organic substances such as lipids, polysaccharides and proteins. Complete oxidation of these products results in the production of carbon dioxide, water and ammonia. Initially the organic substrates are reduced then broken down by dehydrogenation

reactions resulting in the production of a reduced co-enzyme; NADH, some oxidised products and a small number of ATP molecules. The NADH is then oxidised by oxygen, modulated by a number of coupled enzymes which form an electron transport chain. It is from the release of energy in this chain that most of the ATP required for body function and activity is produced.

ATP is formed during both parts of this process (Fig 1:2). When oxygen is not available the NADH cannot be oxidised along the electron transport chain so this process stops. However, the initial part of the sequence (glycolysis) will stop also since hydrogen bound in NADH cannot accumulate due to the limited amount of NAD^+. It is obvious that energy can only be produced in the absence of oxygen if hydrogen can be stored in another way, or when another oxidizing agent can replace oxygen at the end of the electron transport chain. By far the most important method of storing hydrogen is by the production of lactate, however the efficiency of this method is only 6% of the energy produced in complete oxidation. Several alternative pathways have been proposed by Hochachka (1973) resulting in different end products such as propionate, succinate and alanine. Though these end products have been shown in invertebrates they have been proven to be quantitatively unimportant in vertebrates (Burton & Spehar, 1971; Johnston, 1975a). Recently it has been suggested that ethanol may be an important end product in carp exposed to complete anoxia (Shoubridge & Hochachka, 1981; Johnston & Bernard, 1983). An anaerobic oxidisation of the electron transport chain has been described in certain anaerobic bacteria which utilize sulphur as the electron acceptor and produce H_2S as an end product. A replacement for oxygen has also been found in the helminth Ascaris lumbricoides. ATP can be produced by ascaris mitochondria with malate as a substrate, resulting in the accumulation of pyruvate and succinate.

Though it appears that glycolysis provides most of the energy needs of a fish during hypoxia there is evidence that additional pathways operate in some species. During anoxic excursions L-alanine and succinate were observed to accumulate as end products in the red muscle of Salmo gairdneri (Johnston, 1975b). Glycolysis did increase but only in the white muscle and glycogen stores decreased by 70 - 85% in both types.

Glycolytic rates calculated from lactate accumulation cannot account for the sustained anoxic tolerance in goldfish and other carp species. Blâzka (1958) and Van den Thillart (1977) have shown CO_2, produced through the pathway of partial lactate oxidation - lactate → pyruvate → acetyl CoA + CO_2, to be an important end product in anoxic goldfish. Van den Thillart et al. (1976) found increases in the concentrations of alanine, creatine, CO_2 and ADP in completely anoxic goldfish. The concentrations of ATP and creatin triphosphate decreased while AMP, glutamate and volatile fatty acids did not change significantly. Hochachka (1975) has described several possible biochemical pathways resulting in end products such as succinate, isobutyrate, isovalerate, acetate, propionate, proline and alanine though these have yet to be proven quantitatively important in fish.

It therefore seems that in the face of low oxygen availabilities a complex integration of responses at the organismic, cellular and molecular levels are activated. The acute responses are largely organismic and involve changes in gill ventilation and blood circulation whereas the chronic ones appear to be predominantly cellular and molecular, involving changes in erythrocytic organic phosphates and enzymatic activity.

The biochemistry of the effects of hypoxia and anoxia on fish muscle are reasonably well documented however the effects of these parameters on the actual structure of the muscle has never been

examined. This study examines the effects of hypoxic acclimation on the oxygen consumption and ultrastructure of the red and white muscles of three species of fish with very different ecological habitats and lifestyles, and tries to relate the changes found to the natural ecology of the species.

Figure 1:1 Diagrammatic representation of the general characteristics of the three main fibre types found in the teleosts.

Location : The shading represents the general position of the particular fibre type in the myotome.

Histochemistry : SDH. The intensity of shading represents the relative histochemical staining reactions for succinic dehydrogenase, a mitochondrial marker enzyme.

ATPase. The intensity of shading represents the relative histochemical staining reactions for Ca^{2+} activated ATPase without any pre-incubation.

Innervation : Type of innervation of different fibre types

Ultrastructure T.S. : General ultrastructure and capillary supply of fibres in transverse section.

Ultrastructure L.S. : General ultrastructure of myofibrils in longitudenal section.

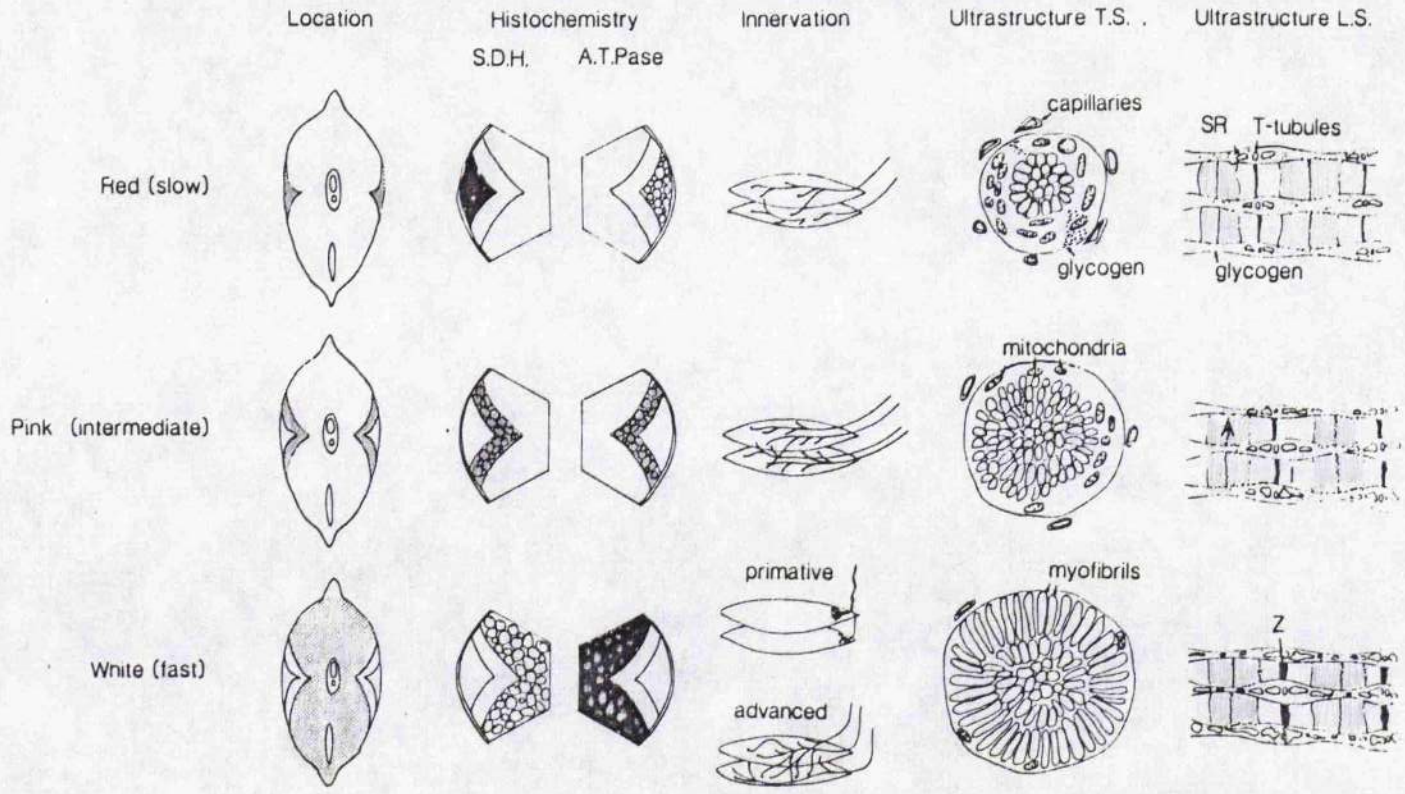
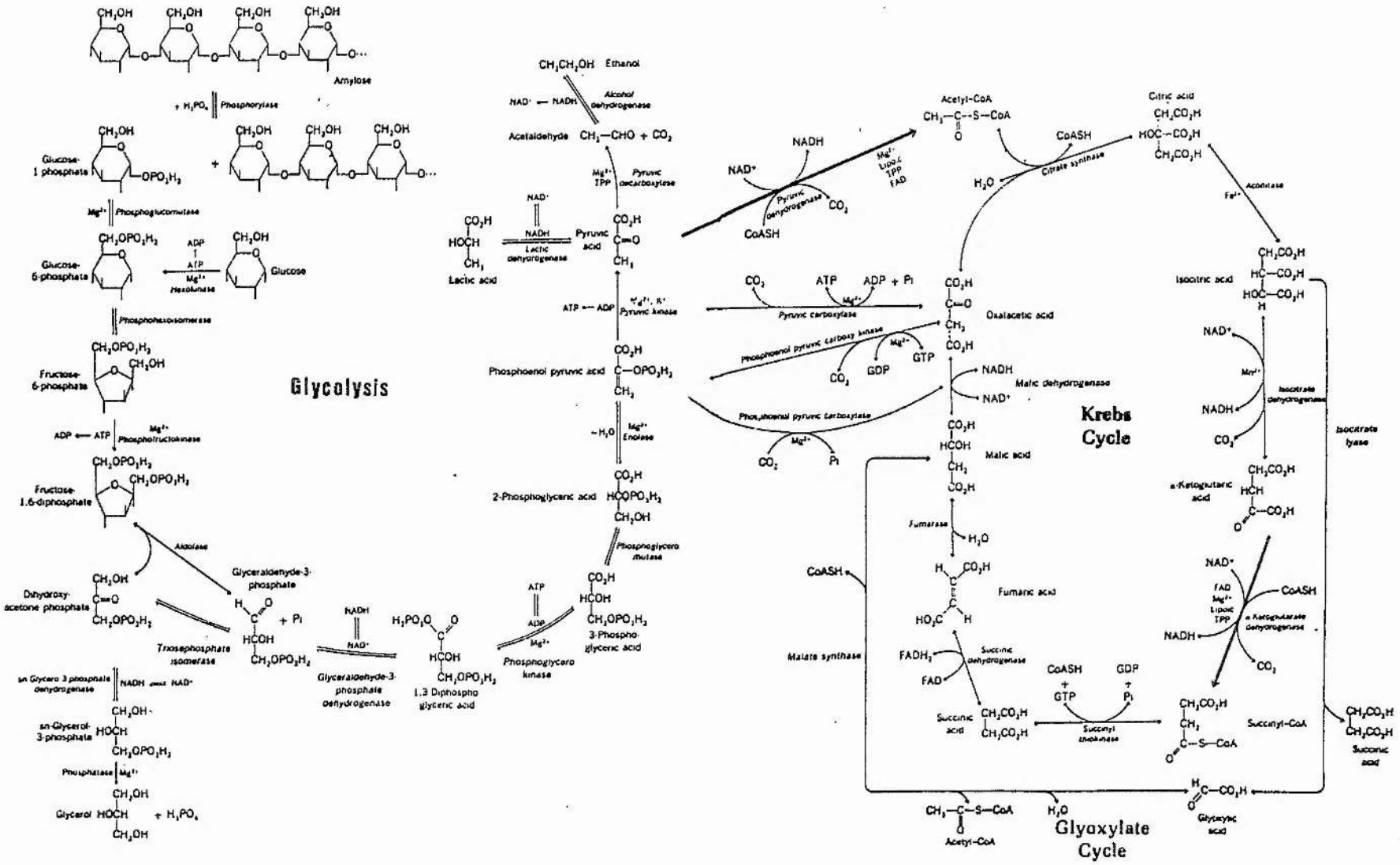

Location
Histochemistry
S.D.H.
A.T.Pase
Innervation
Ultrastructure T.S. .
Ultrastructure L.S.
Red (slow)
capillaries
glycogen
SR
T-tubules
glycogen
Pink (intermediate)
mitochondria
White (fast)
primative
advanced
myofibrils
Z

Fig 1.1

Figure 1:2 Diagram showing the main steps in intermediary metabolism leading to the production of ATP. The glycolytic cycle can occur without oxygen however krebs cycle cannot.


Amylose
Glucose-1-phosphate
Glucose-6-phosphate
Glucose
Fructose-6-phosphate
Fructose-1,6-diphosphate
Dihydroxy-acetone phosphate
Glyceraldehyde-3-phosphate
sn-Glycerol-3-phosphate
Glycerol
Glycolysis
Ethanol
Acetaldehyde
Lactic acid
Pyruvic acid
Phosphoenol pyruvic acid
2-Phosphoglyceric acid
1,3 Diphospho glyceric acid
3-Phospho-glyceric acid
Acetyl-CoA
Citric acid
Oxalacetic acid
Malic acid
Fumaric acid
Isocitric acid
α-Ketoglutaric acid
Succinyl-CoA
Succinic acid
Glyoxylic acid
Krebs Cycle
Glyoxylate Cycle

CHAPTER 2

MATERIALS AND METHODS

Fish

Three species of fish with very different lifestyles were chosen as experimental animals. These species could expect to experience hypoxia in their natural environments.

The Tench, Tinca tinca L. (Fig. 2:1 (b)) is a member of the Cyprinid family. It has a rather thickset elongate body, deep tail and very small, deep set scales covered by a heavy mucus. The colour is a deep greeny brown on the dorsal surface with bronze sides and a yellowish belly. They may attain a maximum length of 70 cm and weight of around 8 Kg. The tench is found throughout Europe inhabiting slow flowing rivers and shallow lakes and feeding on small, bottom invertebrates. During the cold part of the winter they stop feeding, bury themselves in the mud, and hibernate. It is during this period that they would be likely to experience natural hypoxia due to limited oxygen supplies in the mud and a decrease in respiration during hibernation (Wheeler, 1978).

The Crucian carp, Carassius carassius L. (Fig. 2:1 (a)) is also a member of the Cyprinid family. The body is deep and high backed with large scales. The natural colour is a yellowish brown, the back being darker with a greenish iridescence and the belly yellowish. They can reach sizes of 50 cm and 1.8 Kg but are usually much smaller. The

Crucian carp is found wild in western, central and eastern Europe in slow flowing rivers and marshy, shallow lakes and ponds. It feeds on plants and small invertebrates. This species is well known for its tolerance of low oxygen levels which often occur due to respiration of plants in its overgrown environment and ice locking of ponds during winter (Blâzka, 1958; Wheeler, 1978).

The Catfish, Clarias Mossambicus (Richter). (Fig. 2:1 (c)) is a member of the family Clariidae. The body is elongate with the dorsal fin stretching the length of the body. Colour varies with habitat but is usually grey brown with a paler cream belly. Clarias are found throughout East Africa and Asia and can reach sizes of 120 cm and 38 Kg. They are omnivorous, feeding on small invertebrates and vegetation and generally inhabit inshore waters and marshy swamps. During the dry season oxygen tensions in these waters are often greatly reduced as a result of rotting vegetation and evaporation. Clarias are obligate air breathers and obtain oxygen from the atmosphere using special supra branchial organs. (Greenwood, 1958).

The carp and tench were obtained during October 1980 from Stanbridge Trout Fisheries, Rochford, England and the catfish from Lake Victoria, Kenya during June 1981.

Acclimation to hypoxia

In the case of carp and tench, the animals were separated into two groups. One group was kept in aerated water (P_{O_2} 120 - 140 mm Hg) (Normoxia) (Airsaturated water has a $P_{O_2} \approx$ 155 mm Hg or 21 KPa.)

whilst the other group was maintained under hypoxic conditions (P_{O_2} 10 - 15 mmHg) as described below.

Both groups of fish were held for two months in identical 200 litre tanks of filtered, recirculating freshwater at 15 ± 0.2°C under a photoperiodic regime of 12h light: 12h dark. Fish were fed daily on a balanced diet of commercial fish pellets supplemented with chopped earthworms and pigs liver.

The apparatus for maintaining reduced environmental oxygen tensions consisted of an open system of poorly aerated water which was passed through a 9ℓ capacity filter containing layers of coarse gravel, glass wool and activated charcoal. Filtration flow rate was approximately 0.5ℓ/min. A second pump circulated water from the main tank to a mixing chamber (MC) (Fig. 2:2 (a)) at a flow rate of 3ℓ/min. The mixing chamber (5ℓ capacity) contained an oxygen electrode and the outlet of a small diaphragm air pump connected to an oxystat. The desired oxygen tension was set with a multi-turn pot to give a reference voltage to which the outlet of the O_2 electrode (Orion Research Model No. 97-08) was compared (Fig. 2:2 (b)). When measured oxygen tension fell below the "set" level the diaphragm pump was activated pulsing a stream of air into the mixing chamber.

Proportional control of the pump was achieved by burst firing of a triac (Fig. 2:2 (b)). P_{O_2} was displayed on a digital voltmeter. Fish were prevented from swimming into the upper third of the tank by a rigid wooden framed net (B). Gas exchange at the surface

of the water was reduced by sheets of thin polythene. Temperature was maintained by means of glass heat exchangers connected to a flow heater/cooler (Grants Instruments, Cambridge). The filter medium contained activated charcoal, which was changed weekly. Oxygen levels were reduced slowly over two weeks to 1.5 KPa ($\pm$ 0.5 KPa) and maintained at that level for six weeks. The other group of fish was maintained in aerated water at an oxygen level of 17.6 KPa. Compared to fish at normoxia those acclimated to hypoxic conditions consumed around five to six times as much food. Mean bodyweight was not changed following six weeks hypoxia and the fish remained active constantly searching the tanks for food.

The experiments on the catfish, Clarias Mossambicus (Richter) were performed in the Department of Animal Physiology, Nairobi, Kenya (altitude 5,600 ft) by the kind permission of Prof. G.M.O. Maloiy. As it was not possible to transport the equipment for lowering oxygen tensions to Kenya a simpler method was devised and used.

The catfish were divided into four groups of four and placed in small aquaria (60 x 25 x 25 cm). Except for the front panels, the sides of the tanks were covered with opaque plastic sheeting. Fish with dissimilar characteristics eg. spots and scars, appearance of fins etc., were chosen for each tank such that it was possible to identify and observe individual catfish. The water in two of the tanks was well aerated and had a P_{O_2} of around 15.3 KPa. Around one third of the water was changed on alternate days. Two other tanks were not aerated and the respiration of the fish was used to reduce the oxygen tension. Water was circulated between

the tanks and passed through an activated charcoal filter (6.5ℓ capacity) to remove waste products and uneaten food. Gas exchange at the surface of these tanks was reduced by pieces of polythene sheeting. The P_{O_2} of the water in the hypoxic tanks fell over 24h until it reached a steady level of 2.4 KPa (range 1.5 - 3.2 KPa). Fish were acclimated for 27 days to either aerated or hypoxic water under natural daylight (12h light : 12h dark) and at room temperature (20°C). The temperature in the laboratory was almost constant ($\pm$ 2°C) over this period. Fish were fed daily on tropical fish food flakes supplemented by chopped pigs liver.

Measurement of Routine Oxygen Consumption

Tench: Fish were transferred to opaque flow through respirometer boxes (6 x 6 x 20 cm) 24 - 36h before measurements of oxygen consumption were made. Oxygen consumption was determined by measuring flow rate (80 - 160 mls/min) and the difference between the oxygen levels of the water entering and leaving the box (Johnston, 1975). P_{O_2} was measured using a Radiometer Oxygen analyser (PHM 72 Mk II). Samples were taken at 15 min intervals and an average value of oxygen consumption calculated for an 8h period. Water was pumped through the boxes from the acclimation tanks (Fig. 2:2 (a)) so that oxygen consumption could be determined at P_{O_2} s of either 1.5 or 17.6 KPa. The boxes were partially submerged in the acclimation tanks to maintain temperature at 15°C. Some other measurements of oxygen consumption were made using closed respirometers of 6.5ℓ capacity immersed in the acclimation tanks. Serial water samples were analysed for P_{O_2} over 3-4h. All measurements were made after at least six weeks acclimation to

hypoxia or normoxia.

Carp: Fish were transferred to opaque closed respirometers of approximately 1ℓ capacity 24h before measurements were begun. The tops were left off and the water gently aerated. At the start of the experiments the respirometers were closed and the respiration of the fish allowed to reduce the P_{O_2} of the water. Oxygen consumption was determined during progressive hypoxia by taking water samples every 20 mins. Experiments were performed using both single fish and groups of fish. All measurements were made after at least six weeks acclimation to hypoxia or normoxia.

Catfish: Routine oxygen consumption was measured, as described by Hughes and Singh (1971) using a closed respirometer which consisted of a water filled chamber of 6.5ℓ and an air chamber of 125 mls (Fig. 2:3). Fish were transferred to the respirometer containing either aerated or hypoxic water at least the night before any measurements were made. During this time the fish remained undisturbed and had free access to the atmosphere. At the start of the experiment the lid was placed on the respirometer so that the fish could only obtain air through a small opening (3 cm^2) to the air chamber (Fig. 2:3). Since the water and air were in direct contact the fish were able to locate the opening and air-breath normally throughout the experiment. Over the time period of the experiments the diffusion of oxygen across the opening was not significant compared to changes due to the respiration of the fish. Samples of air and water were collected in syringes at intervals of between 45 and 60 mins. The P_{O_2} of the water samples was measured using a Radiometer PHM 72 oxygen analyser and the % O_2 content of air samples analysed with a Taylor Oxygen meter. Measurements of respiration rates of

fish from the hypoxic tanks were started after 18 days of acclimation to reduced oxygen and continued until day 27 when all fish were sacrificed. The initial P_{O_2}'s in the respirometers were adjusted by bubbling either O_2 or N_2 to values around 1.4 KPa above that in the acclimation tanks. At least two experiments were carried out on each fish.

Opercular frequencies were counted for periods of 2-5 mins at intervals throughout the experiment.

Two experiments were also carried out in a flow through respirometer (20 x 8 x 8 cm) in which the fish had no access to the air. Aerated water was continuously circulated (80 - 110 ml/min) through the box and inflow and outflow P_{O_2} measured at 30 min intervals for 6-8 h.

All respiration experiments were carried out at room temperature (20°C).

Due to the fact that the fish had been chosen to be individually identifiable it was possible to time the intervals between air breaths for each fish. Intervals were timed for each fish in the hypoxia tanks for periods during the 36 h following the reduction of the oxygen tension to 2.4 KPa. Further measurements of air breathing frequency were made after 10 and 13 days hypoxia. Air breathing frequencies of fish in the aerated tanks were measured on days 16 and 17.

Histochemistry

Fish were stunned by a blow to the head then pithed. The types of muscle fibre present and their distribution was studied by staining serial frozen sections for succinic dehydrogenase (SDH ase) activity, glycogen, and myofibrillar ATP ase activity following alkaline pre-incubation.

Samples of muscle (≈ 5 mm diameter) were excised from the lateral line region near the longitudenal mid point of the body. The blocks were mounted on chilled cryostat chucks in an inert embedding medium (OCT compound, Lamb, London) and frozen in iso-pentane (2-methyl butane) cooled to near its freezing point (-150°C) in liquid nitrogen. Blocks were then equilibrated to -20°C and frozen sections 8-10 μm thick were cut on a Pearse Slee cryostat and mounted on dry coverslips.

Staining Procedures

All incubations were carried out at room temperature (≈20°C). Sections were stained on coverslips and mounted on glass slides in glycerin jelly.

Sections were stained for glycogen by the periodic acid Schiffs (PAS) method as described by Pearse (1960). In view of the high glycogen content of tench muscle a 1 min treatment in 1% aqueous periodic acid was found to be sufficient prior to staining with Schiffs reagent. The catfish required a 15 min treatment in periodic.

Succinic dehydrogenase activity was localised by incubating sections for 30 - 60 mins in a solution containing 80 mM sodium succinate, 50 mM potassium phosphate buffer, pH 7.4, with 1 mg/ml nitroblue tetrazolium (NBT) (Sigma Chemical Co. Ltd., London) as the electron acceptor.

Sections were stained for Ca^{2+} activated ATPase activity following alkaline preincubation at pH10.4 (Johnston et al., 1974). Pre-incubation was for 2 - 20 mins in a medium of, for the tench, 1 mM$CaCl_2$, 100 mM Tris-HCL (pH 10.4) and for the catfish 18 mM $CaCl_2$, 50 mM 2-amino-2 methyl-1 propanol (pH 10.4) (221 buffer, Sigma Poole, Dorset). Sections were subsequently incubated in, for the tench, an incubation medium containing 5 mM ATP, 1 mM $CaCl_2$, 30 mM KCl, 10 mM Tris HCL (pH 9.4) and for the catfish, 20 mM KCl, 8 mM $CaCl_2$, 30 mM ATP and 80 mM 221 buffer (pH 9.2) for 15 mins, washed in water and successively treated with 3% Cobalt chloride (3 min.) washed in water again and finally stained with 1% ammonium sulphide solution. Controls were performed in which ATP was omitted from the incubation medium. Preincubations of 1 - 5 mins result in inactivation of slow muscle myosin. Longer periods of alkaline preincubation result in a progressive loss of staining of fast glycolytic fibres, whereas fast aerobic fibres stain intensely for myofibrillar ATPase even after 20 mins of preincubation at alkaline pH.

Staining for acetylcholinesterase activity.

Muscle endplates and pre-terminal axons were localised by staining fibres for acetylcholinesterase activity. Small bundles (3-4 myotomes, $2mm^2$ x 20mm long) were dissected complete with myoseptal attachments from the mid epaxial muscalature below the

dorsal fin and pinned on to cork strips at their resting lengths in situ. Samples were fixed at room temperature for 2 - 8 hrs in 10% formalin, 100 mM acetate buffer, pH 5.2, then thoroughly washed in distilled water. The strips were subsequently incubated for 6 - 8 hrs at 37°C in a freshly prepared solution containing 2.5% $CuSO_4 5H_2O$ (0.2 ml); 3.7% glycine (0.2 ml); 0.2N sodium acetate buffer, pH 5.2 and the clear-supernatent from 15 mg acetylcholine iodide, 0.3 ml of 2.5% copper sulphate and 0.7 ml water (Naik, 1963). Samples were then stained in 1% ammonium sulphide, washed in water and cleared in glycerol for48 - 72 hrs at room temperature. Small fibre bundles were teased out under a binocular microscope (X7) and mounted in glycerol on glass slides.

Electron Microscopy

Fixation of muscle samples.

Small strips (3 x 5 x 20 mm) of superficial red muscle were dissected from the myotomes immediately posterior to the dorsal fin in the region of the lateral line nerve (Fig. 2:4 (a)). White muscle strips of similar size were excised from the underlying muscle. Care was taken to exclude the fibres on the boundary between the red and white muscle zones as these represent a distinct fibre type. Strips were pinned to cork board at their resting lengths in situ and immersed in 3% gluteraldehyde, 0.1M phosphate buffer, pH 7.2 at room temperature. Initial fixation was for 2 - 5 hr. Subsequently, small fibre bundles (100 - 150 slow fibres and 20 - 50 fast fibres) were dissected from the superficial layers of the strips and left overnight in a fresh change of the same fixative. Fibre bundles were then washed in 0.12M phosphate buffer, pH7.2 at room temperature then post fixed in 1% osmium tetroxide, 0.12M phosphate buffer,

dehydrated in a series of alcohols up to 100%, cleared in epoxypropane and embedded in araldite resin, CY 212) EM Scope, Trent, England). Semithin (1 μm) and ultrathin (400Å) sections were cut on an OM U2 Reichert Ultramicrotome. Semithin sections were stained in 1% toluidine blue stain and viewed under a light microscope (X10-100) to assess the orientation and potential of the block. Ultrathin sections were mounted on formvar-coated, 150 mesh, copper grids, double stained with uranyl acetate and lead citrate and viewed with a Philips 301 electron microscope.

Morphometric Techniques

Quantification of muscle fibre components

The numbers involved for the separate species and groups of fish studies are shown in Table 2:1.

Equal numbers of fish were sampled for each condition (eg acclimated to either aerated or hypoxic water). For each fibre type (red or white) a number of transversly and longitudenally orientated blocks were prepared. The blocks from each fish were kept separately in the case of the carp and tench and randomly mixed for the catfish.

An extremely detailed investigation of the composition of the red and white fibres in aerated and hypoxic water acclimated fish was made only in the case of the tench. For the carp and catfish the method described below was used to determine the volume fraction occupied by the mitochondria only.

The volume fraction occupied by myofibrils, mitochondria and other cellular components was determined from micrographs of transversely

orientated fibres. For each group, sections were cut from 25 - 35 blocks that were selected at random (eg red fibres from fish acclimated to aerated water). Each section contained approximately 50 - 80 red (slow) fibres or 20 - 30 white (fast) fibres. From each of these sections three to four whole fibres were photographed at random (magnification x 2000 - 3400). In the case of white fibres it was often necessary to photograph two or three overlapping fields in order to reconstruct photomicrographs of the whole fibre. Negatives of micrographs of whole fibres were projected so that the image overlaid a square test grid. Reconstructed photomicrographs were placed under a transparent acetate test grid. The projected images or photomicrographs were magnified such that the mean mitochondrial size was similar in all cases (magnification x 2.6 - 4.4). Volume fractions of mitochondria and myofibrils etc. were determined using a point counting method (Weibel, 1980). The test system consisted of a coherant grid composed of discrete line segments (1 cm) with endpoints arranged in a regular square lattice. Magnification was such that the test lines corresponded to a distance of 2.3 μm on the image which is equivalent to between 1 and 1.5 times the mean diameter of the mitochondria (Mathieu et al., 1980). The volume fractions (V_v) of each component were determined from:

$$V_v = \frac{Pi}{Pt}$$

where Pi = number of points falling on a component and Pt = total number of points falling on the fibre. The area outside a line drawn around the myofibrils was considered to represent the subsarcolemmal zone. Mitochondria were scored as falling either in the subsarcolemmal or intermyofibrillar zones. There were at least 200 test points on each fibre. Areas and perimeters of fibres and the subsarcolemmal

zone were determined from traced outlines of the fibres (magnified x 2.6) using a digital planimeter (Summagraphics Ltd.) interfaced with a P6060 Olivetti mini-computer. The point counting method was found to give > 95% agreement with determinations of V_v using the digital planimeter. In the case of the tench direct counts were made of the numbers of mitochondria per cross section occurring in both the subsarcolemmal and intermyofibrillar zones.

The sarcotubular system was quantified from longitudenal sections for the tench only. A total of 27 micrographs (print x plate magnification 39000 x 50000) were analysed for each subgroup. Blocks from each fish were kept separately and two blocks were selected at random from each subgroup. Only six out of eight fish in each group were analysed giving a total of 24 blocks. Ultrathin sections were cut and the sections photographed such that each plate sampled five to ten myofibrils over a distance of three to four sarcomeres. The areas and perimeters of sarcoplasmic reticulum (S.R.) and of the T-system in each micrograph were determined directly by planimetry of tracings (magnified 2.6 x plate size). Volume fractions (V_v) of the components were calculated as follows:

$$V_v = \frac{Ai}{A_t} \times 100$$

where Ai = individual component profiles
and A_t = projected sample area. Values of V_v for SR and T-system were expressed as a percentage of myofibrillar volume of fibres. These figures were corrected for differences in the volume percentage of mitochondria between the different subgroups. Errors in quantification are the summed areas of the individual tracings.

Reproducability was found to be acceptable at 95%.

Analyses of muscle capillary supply

Sections were cut from approximately 30 transversely orientated blocks per subgroup (eg Hypoxic acclimated tench red muscle). Low power electron micrographs (X570 - 910) were taken using a ¼ plate camera of areas of the section containing a number (5 - 15) of fibres with their associated capillaries. Areas in which the fibres and capillaries had not splayed apart were chosen. The micrographs were then projected through a photographic enlarger at a magnification x 2.6 and the outlines of fibres and capillaries traced onto white cartridge paper.

Fibre cross-sectional areas and perimeters, capillary cross-sectional areas and perimeters and the lengths of contacts between capillaries and fibres were determined directly by digital planimetry using a Summagraphics digitiser inter-faced with an Olivetti P6060 mini-computer. In addition the number of capillaries and fibres were counted and the number of capillaries touching each fibre also counted. The following parameters were measured (replication error $\approx$ 2%):

(A) Number of fibres analysed

(B) Percentage of fibres without direct capillary contact

(C) Number of capillaries in direct contact with fibres analysed

(D) Mean fibre cross-sectional area (μm^2)

(E) Mean fibre circumference (μm)

(F) Volume density of mitochondria (see above)

(G) Average number of capillaries in contact with each fibre

(H) Mean length of contacts between fibre and capillaries (μm)

$\bar{a}$ (c) Mean cross-sectional area of capillaries (μm^2)

$\bar{b}$ (c) Mean circumference of capillaries (μm)

Various other indices of capillarisation were calculated from the measured parameters.

(I) Percentage of fibre circumference in direct contact with capillaries

$$\frac{\text{Contact length between fibre and capillaries}}{\text{Fibre circumference}} \times 100$$

(J) Capillary circumference (um) per um^2 of fibre cross-sectional area

$$\frac{\text{Contact length between fibre and capillaries}}{\text{Fibre cross-sectional area}}$$

(K) 'Area' of capillary wall supplying 1 um^3 of mitochondria

$$\frac{J}{F}$$

N_A (C,F) Number of capillaries per unit volume of muscle fibres (mm^{-1})

$$C/_A \times D \ (mm^2)$$

Statistical Analyses

Data from fish acclimated to aerated or hypoxic water were compared using a one-way analyses of variance for unequal sample numbers.

TABLE 2 : 1

Species, Acclimation group and fibre type	Number of fish studied	Total number of blocks prepared	Total number of blocks cut for analyses	Number of fibres analysed
Tench, aerated, red	8	96 TS 40 LS	25 TS 16 LS	62
Tench, aerated, white	8	96 TS 40 LS	25 TS 16 LS	66
Tench, hypoxic, red	8	96 TS 40 LS	25 TS 16 LS	90
Tench, hypoxic, white	8	96 TS 40 LS	25 TS 16 LS	58
Crucian carp, aerated, red	8	96 TS 40 LS	25 TS	25*
Crucian carp, aerated, white	8	96 TS 40 LS	25 TS	25*
Crucian carp, hypoxic, red	8	96 TS 40 LS	25 TS	25*
Crucian carp, hypoxic, white	8	96 TS 40 LS	25 TS	25*
Catfish, aerated, red	8	48 TS 24 LS	25 TS	25*
Catfish, aerated, white	8	48 TS 24 LS	25 TS	25*
Catfish, hypoxic, red	8	48 TS 24 LS	25 TS	25*
Catfish, hypoxic, white	8	48 TS 24 LS	25 TS	25*

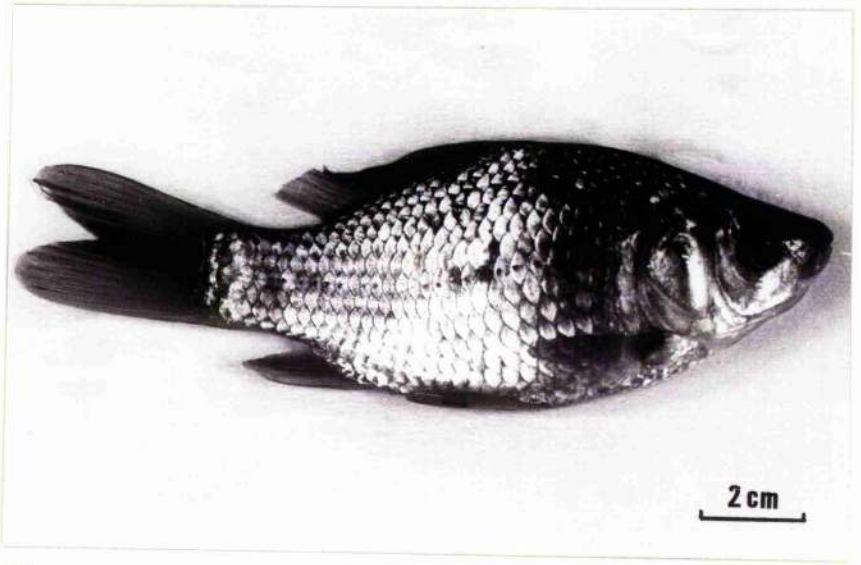
* Analysed for mitochondrial fractional volume only.

Figure 2:1

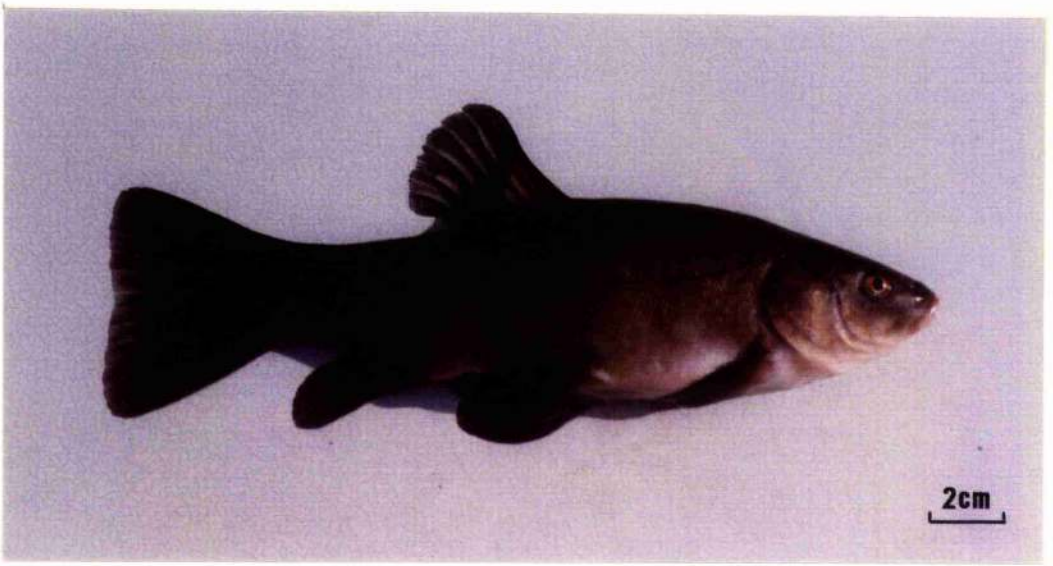
(a) The Crucian carp, Carassius carassius.

(b) The Tench, Tinca tinca.

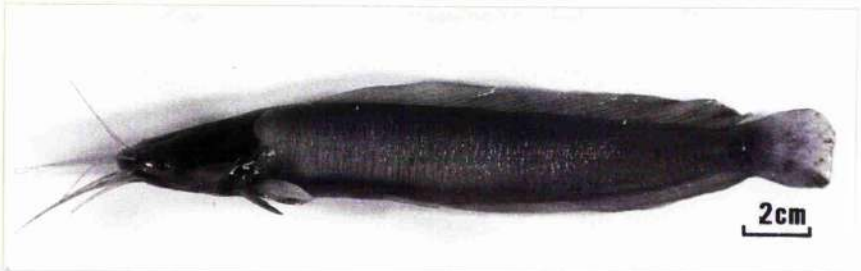
(c) The Catfish, Clarias mossambicus.



a



b

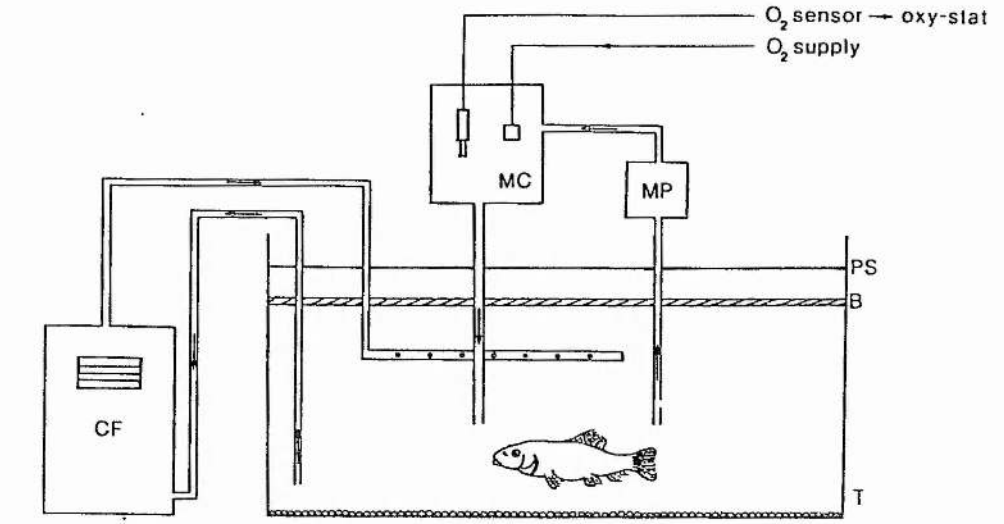


c

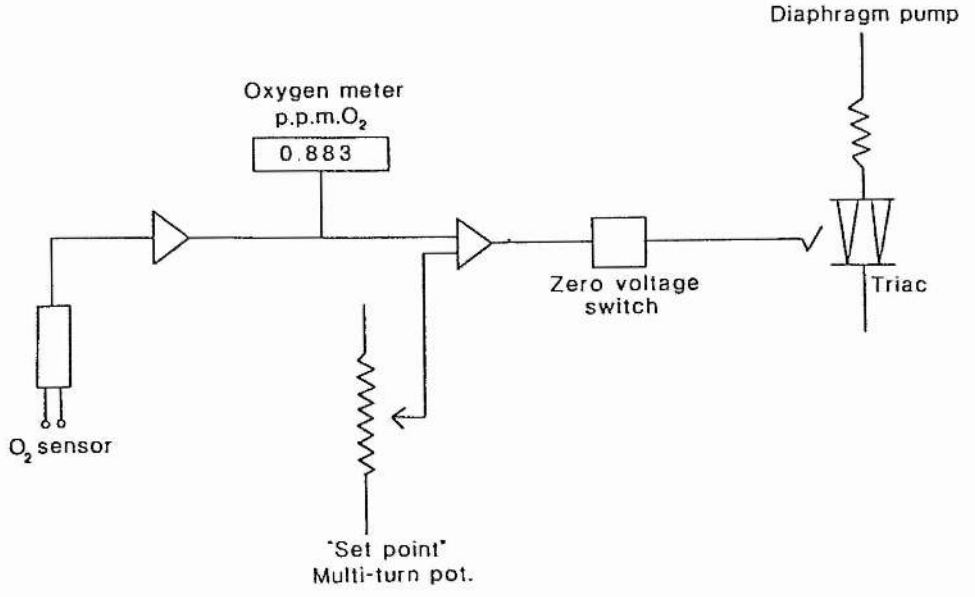
Figure 2:2

(a) The apparatus used to maintain fish at reduced oxygen levels. T tank; CF activated charcoal filter; MP mixing pump; MC mixing chamber; B rigid wooden-framed net; PS plastic sheeting. Temperature control was achieved by means of a series of glass coils in series with a Flow heater/Cooler (Grants Instruments). For clarity, the heat-exchange coils are omitted from the diagram.

(b) Block diagram of the electronic circuit of the oxystat.


O_2 sensor → oxy-stat
O_2 supply
MC
MP
PS
B
CF
T

(a)


Diaphragm pump
Oxygen meter
p.p.m.O_2
0.883
Zero voltage
switch
Triac
O_2 sensor
"Set point"
Multi-turn pot.

(b)

Fig 2.2

Figure 2:3

Closed type respirometer box used for measuring respiration rates of the catfish, Clarias mossambicus.

AC air chamber, 125 mls; WC water chamber, 6.5 litres; SP sampling ports for air and water; BH 2 cm radius hole between the air and water chambers.

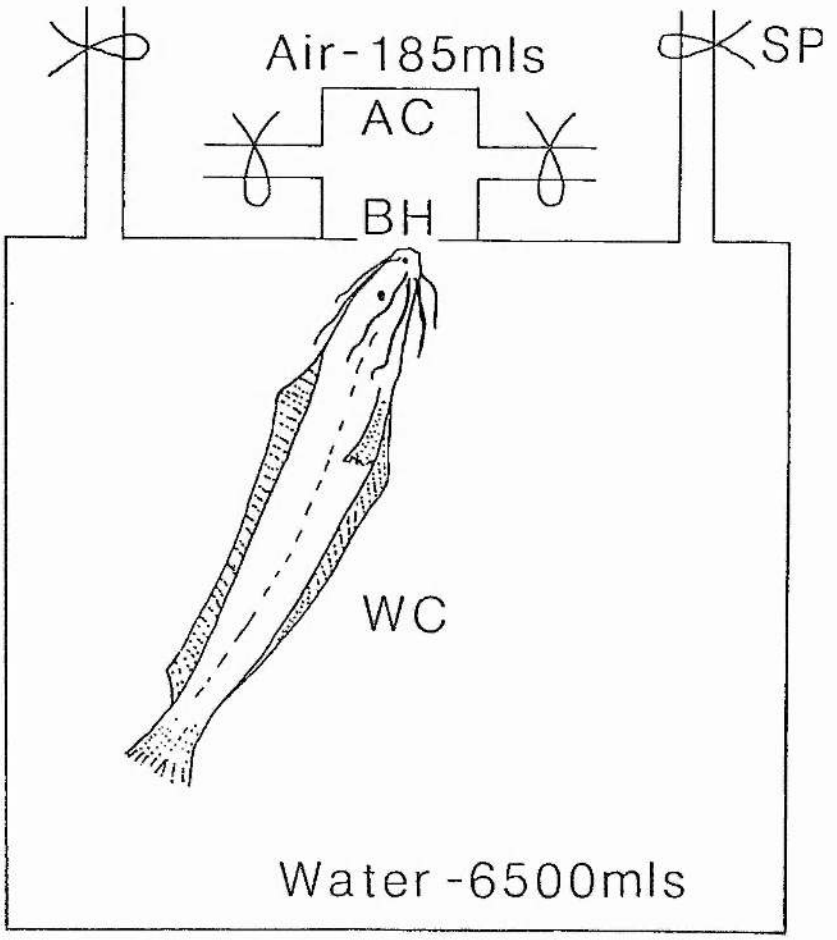
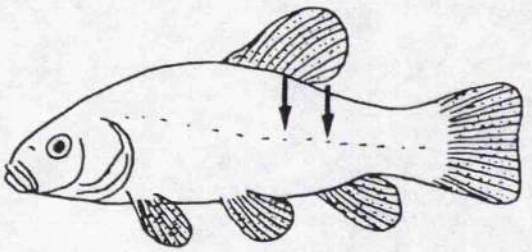


Fig 2.2

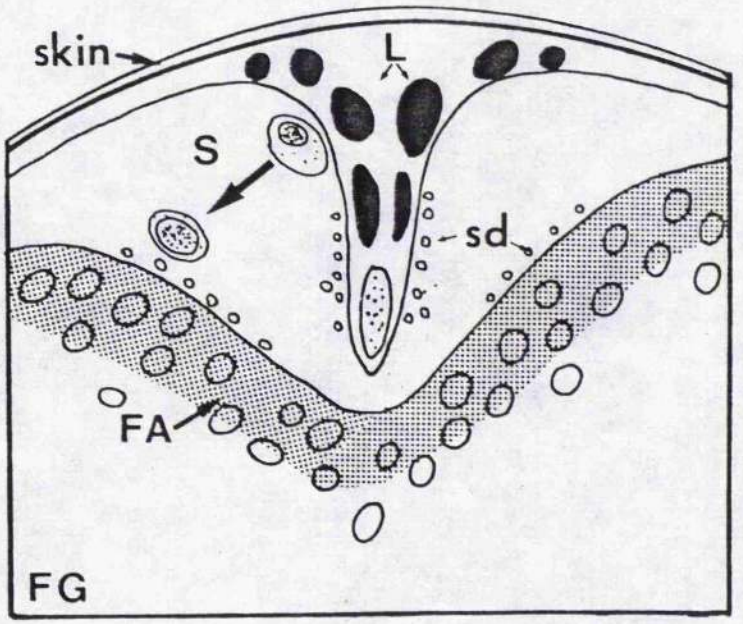
Figure 2:4

(a) The position of the sampling points (indicated by arrows) for fibres used for ultrastructural studies. The same basic sampling area was used in all three species.

(b) A diagrammatic representation of the distribution of fibre types in the myotomal muscle of the tench, Tinca tinca. There is a progressive decrease in the size of the subsarcolemmal zone in slow fibres along a transect from the skin to the outer border of the fast aerobic muscle fibre zone.
L extracellular lipid droplets; FG fast glycolytic fibres; FA fast aerobic fibres; sd small diameter fibres; S slow fibres.



(a)



(b)

CHAPTER 3

OXYGEN CONSUMPTION

Introduction

Many species of fish are able to adapt physiologically to changes in their natural environment, be it changes in temperature, food availability or oxygen levels in water (Johnston, 1981a,1982; Beardall & Johnston, 1983; Johnston & Bernard, 1982 a, b). Few authors have studied the effects of acclimation to low oxygen levels on the respiration and metabolism of fishes (Beamish, 1964; Kutty, 1968b; Van den Thillart, 1977; Lomholt & Johansen, 1978).

Measurements of oxygen consumption provide a method of assessing the effects of hypoxia and acclimation to hypoxia at the organismic level. Though many separate physiological changes may occur such as alterations in gill ventilation, heart rate, oxygen carrying capacities and tissue ultrastructure (see Chapter 1) these generally combine to produce an overall effect on the animal of altering the oxygen consumption rate.

Fry (1947) formulated a hypothesis to explain the relationship between oxygen consumption and environmental oxygen level in fish (Fig. 3:1). He proposed that standard oxygen consumption rate was independent of oxygen concentration whereas active consumption rate was strongly dependent in a hyperbolic way. The level where all consumed oxygen is used in maintenance metabolism (the "level of no excess activity") would be at the intersection of standard and active metabolism, thus, activity would only be possible above this rate. Fry thought that acclimation to hypoxia would result in a shift to lower levels in the active rate of oxygen consumption (Fig. 3:1). Beamish (1964) and

Kutty (1968) showed that in goldfish and rainbow trout active metabolism was not influenced by hypoxia acclimation, whilst standard metabolism was changed in such a way that a single intersection could not be established. Activity was increased and both standard and routine oxygen consumption were decreased following several weeks acclimation to hypoxia.

In contrast to Beamish and Kutty, Van den Thillart (1977) supports Fry's original concept. In his experiments with goldfish minimal routine rates of oxygen consumption, measured at night, showed a remarkable 'non confirmity' with respect to the oxygen content of the water. At low oxygen concentrations he found an increase in consumption rates and concluded that the standard metabolic rate would increase during hypoxia due to the increasing cost of oxygen uptake and that during hypoxia part of the energy used is produced anaerobically, confirmed by lactate increase and oxygen debt.

Studies by Prosser et al. (1957) and Lomholt and Johansen (1978) working with goldfish and common carp show a lower average oxygen consumption rate for hypoxia acclimated fish when measured in aerated water compared to normoxia (aerated water) acclimated animals. During severe hypoxia the hypoxia acclimated fish had a higher oxygen uptake than the normoxia acclimated fish.

Biological literature cites many values of oxygen consumption for various fishes but these are meaningful only for the particular conditions of measurement, thus, it is difficult to compare data given by one author to that of another and tabulated values are subject to necessary qualifications. Rate of oxygen consumption is influenced by activity, temperature, body size, stage in life cycle, season and time of day as well as previous oxygen level exposure and genetic background.

Spoor (1946) found a circadian rhythm correlated with oxygen consumption in active goldfish, as did Van den Thillart (1977). Muscular movement can increase oxygen consumption by up to 15 times its resting

level (Bennet, 1978). Oxygen consumption rises and falls by about 1.5 - 2 times per 10°C in the physiological range (Prosser, 1973).

To enable comparisons to be made, it was important during these experiments to ensure as few variables as possible. "Routine" oxygen consumption, defined as oxygen consumption with uncontrolled but minimum motor activity, was measured for all three species, the only variable between the groups being the oxygen acclimation level. Fish of, as near possible, same age, size, genetic background, and nutritional status were chosen. During the measurements the animals movements were minimised by darkness, quiet and habituation to the respirometer. During acclimation and measurements animals were kept at the same temperature, photoperiodic regime and feeding schedule and measurements were made at the same time of day.

Thus, for a particular set of conditions (defined in Chapter 2), the oxygen consumptions of three different species, differing only in the acclimation oxygen tensions of their environments, have been measured. It can be concluded that the differences found between acclimated groups (acclimated to low or aerated water oxygen tensions) are due to changes in the animals brought about by the acclimation period.

Materials and Methods

The tench, Tinca tinca L.

Twelve fish of mean length ($\pm$ SE) 20.0 $\pm$ 0.5 cm were divided into two groups and acclimated to P_{O_2} of either 1.5 or 17.6 KPa, (hypoxic or normoxic), as described in Chapter 2.

The Crucian carp, Carassius carassius L.

Twelve fish of mean body weight ($\pm$ SE), 16.1 $\pm$ 1.2 g were acclimated to aerated water ($P_{O_2} \approx$ 17.6 KPa), and six fish of mean body weight

hypoxia linearly parallel with water P_{O_2} to 16.2 mls O_2 / Kg bodyweight /h at P_{O_2} of 1.5 KPa. (Fig. 3:3). Hypoxia acclimated fish in aerated water have an oxygen consumption rate of 82.4 $\pm$ 6.7 mls O_2 / Kg bodyweight / h. During progressive hypoxia the acclimated fish were able to maintain this consumption rate until P_{O_2} of the water reached 6.9 - 4.2 KPa after which it fell rapidly. At $P_{O_2} \approx$ 1.5 KPa respiration rate was reduced by around 65% in the hypoxia acclimated group and 80% in the aerated water acclimated group and standard oxygen consumption of the hypoxia acclimated group was approximately twice that of the aerated water acclimated group.

The catfish, Clarias mossambicus

The results obtained for catfish respiration are not directly comparable with those of the other species due to the fact that Clarias are obligate air breathers even in aerated water. Experiments were carried out on two fish in aerated water ($P_{O_2} \approx$ 16 KPa), flow through type respirometers with no access to air to establish survival times. A smaller animal of bodyweight 25.6 g survived 52 hrs. during which time the ventilatory movements increased markedly, opercular frequency went up from 24 to 82 per minute and the average respiration rate was 44.9 mls O_2 / Kg / h. The second fish of bodyweight 48.6 g survived only 24 hrs., its opercular frequency also being raised to 82 per minute and its mean respiration rate to 76.6 mls O_2 /Kg / h.

Measurements of the intervals between air-breaths of fish left undisturbed with free access to air show a large variation, not only between fish but for the same fish (Fig. 3:4). On initial exposure to hypoxia (first 36 h) mean air breathing frequency was 6.1/h, not significantly different from fish in aerated water (Table 3:2). Acclimation to hypoxia ($P_{O_2} \approx$ 2.4 KPa), for 11 - 13 days resulted in an

($\pm$ SE), 15.7 $\pm$ 2.9 were acclimated to hypoxic water ($P_{O_2} \approx$ 1.5 KPa), as described in Chapter 2.

The catfish, Clarias mossambicus Richter

Twenty fish of mean body weight ($\pm$ SE), 50.0 $\pm$ 5.3 g were divided into two groups and acclimated to hypoxic or aerated water as described in Chapter 2.

The methods of measurement of oxygen consumption were as detailed in Chapter 2.

Results

The tench, Tinca tinca

Measurements of the oxygen consumption of tench acclimated to aerated water, made in aerated water, show similar values for both the closed and the flow through type respirometer boxes (Table 3:1; Fig. 3:2a). On exposure to acute hypoxia in a closed respirometer, oxygen consumption of non acclimated animals fell from 32.7 $\pm$ 0.5 mls O_2 / Kg bodyweight / h (mean $\pm$ SE mean) to 10.8 $\pm$ 0.8, a 66% decrease ($P < 0.01$) (Table 3:1; Fig. 3:2a). Following six weeks acclimation to hypoxia, oxygen consumption in hypoxic water had risen slightly to 15.6 $\pm$ 1.7 mls O_2 / Kg bodyweight / h (mean $\pm$ SE mean) or a 49% decrease in oxygen consumption.

The Crucian carp, Carassius carassius

The oxygen consumption of carp acclimated to aerated or hypoxic water, during progressive hypoxia is shown in Fig. 3:3. In aerated water, normoxic acclimated carp show a mean oxygen consumption ($\pm$ SE mean) of 75.7 $\pm$ 4.1 mls O_2 / Kg bodyweight / h which fell during progressive

increase in the air breathing frequency to 8.1/h and a decrease in the time interval between breaths (Table 3:2; Fig. 3:5).

Table 3:3 gives the results for routine respiration rates for fish in closed respirometers. In aerated water acclimated fish measured in aerated water 24.6% of total oxygen consumption was attributable to air breathing, however on exposure to acute hypoxia this rose to 77.5% (Table 3:3; Fig. 3:2b). Acute hypoxia caused a 54% decrease in total respiration largely as a result of an 84% decrease in aquatic respiration rate from 64.6 to 10.4 mls O_2 / Kg / h ($P < 0.001$; Table 3:3; Fig. 3:2b).

Acclimation to hypoxia ($P_{O_2} \approx 2.4$ KPa) for 27 days resulted in a 22% increase in total respiration rate when compared to fish subject to acute hypoxia (Table 3:3; Fig. 3:2b) ($P < 0.05$), largely due to a 164% increase in aquatic respiration from 10.4 to 25.5 mls O_2 /Kg / h ($P < 0.01$).

Ventilatory movements of fish in both aerated water and when exposed to acute hypoxia were of similar frequency (25.4 ± 2.1 min^{-1}), but during acute hypoxia amplitude was markedly increased. After acclimation to hypoxia opercular frequency had increased to 33.3 ± 2.2 min^{-1} and amplitude was substantially greater.

Discussion

Two points, in common to all three species, are confirmed by the results of these experiments.

1. Oxygen consumption in hypoxic water is depressed to levels substantially below that measured in aerated water, regardless of acclimation group.

2. After acclimation to hypoxia, oxygen consumption in hypoxic

water is higher than in non acclimated groups.

Comparable responses to decreased oxygen levels have been observed in other metabolic conformers including goldfish, Carassius auratus. (Van den Thillart, 1982), oyster toadfish, Opsanus tau (Hall, 1929), brown bullhead, Ictalurus nebulosus (Marvin & Heath, 1968) and carp, Cyprinus carpio (Lomholt & Johansen, 1978).

Lomholt and Johansen (1978) showed that at low oxygen tensions the oxygen consumption of hypoxia acclimated fish was 30 - 40% higher than normoxia acclimated fish. Distinct differences in the breathing pattern were observed between fish in aerated water and hypoxic water. Breathing in aerated water showed a pattern of 4 - 6 breaths followed by a 10 - 20 s apneic period. Hypoxia induced continuous breathing, gill ventilation rising from an average of 195 mls / Kg / min to 1122 mls / Kg / min (Lomholt & Johansen, 1978). After a period of acclimation to hypoxia gill ventilation fell to around 800 mls / Kg / min and the percentage of available oxygen extracted rose to 85% compared to 72% in non-acclimated fish.

Both crucian carp and tench show similar results to those of the common carp. Acclimation to hypoxia in all three species causes a significant increase in oxygen consumption in hypoxic water relative to non-acclimated fish (Table 3:1; Fig. 3:2a, 3:3; Lomholt & Johansen, 1978).

The increased oxygen capacity of hypoxia acclimated fish could result from several factors including increase in gill ventilation, changes in gill perfusion and altered respiratory properties of the blood through increases in haemoglobin - O_2 affinity (see Chapter 1). Weber and Lykkeboe (1978) have shown a striking increase in blood-oxygen affinity in carp, Cyprinus carpio, acclimated to hypoxia which can be correlated with decreases in concentrations of the red cell nucleoside triphosphates.

It can be deduced due to the increased oxygen consumption in tench and Crucian carp after acclimation to hypoxia that a series of factors must be combining to produce adaptation in the animals to the adverse conditions of their environment.

Direct comparisons between oxygen consumptions in aerated water of Crucian carp and tench show that routine oxygen consumption of tench is approximately half that of Crucian carp. Acute exposure to hypoxia in non-acclimated fish led to a drop of V_{O_2} to 33% of that in aerated water for the tench and 21% for the Crucian carp. Following several weeks acclimation V_{O_2} in hypoxic water rose to 48% that in aerated water for the tench and 40% for the Crucian carp.

These differences are likely to be due to species variations and may account for some of the differences in ultrastructural results obtained. This is discussed in Chapter 6.

A reduction in spontaneous locomotary activity has been observed in several species as a result of acclimation to hypoxia (van den Thillart, 1977; Lomholt & Johansen, 1978). It seems likely that routine oxygen needs can be met comfortably by hypoxia acclimated fish. Lomholt & Johansen (1978) showed that the lowest routine oxygen consumption levels for common carp measured in aerated water were comparable to the highest rates of consumption of hypoxia acclimated individuals in hypoxic water. However, the aerobic scope for activity must be reduced in hypoxic water due to the increased cost of obtaining oxygen.

Experiments on the tench, Tinca tinca, acclimated to either hypoxic of aerated water have revealed a doubling of the maximal activities of phosphofructokinase (PFK) in slow muscle and of PFK, pyruvate kinase and lactate dehydrogenase in liver of fish acclimated to hypoxic water (Johnston & Bernard, 1982b). These results suggest an increase in the capacity for anaerobic energy production which may reflect a reduced

threshold for anaerobic metabolism during activity (Johnston & Bernard, 1982b).

Due to its ability to air-breathe the catfish is discussed separately.

The responses of Clarias mossambicus to acute hypoxia are very similar to those of the closely related Asian species Clarias batrachus (Singh & Hughes, 1971). When measured in aerated water C.mossambicus obtained 25% of its total oxygen needs from the air whereas C.batrachus obtains around 58% (Singh & Hughes, 1971). On exposure to acute hypoxia total respiration fell by 54% in C.mossambicus and 74% in C.batrachus and both species showed a decrease in the interval between air-breaths and an increased reliance on aerial respiration (Moussa, 1957; Singh & Hughes, 1971). The absolute differences between the two species are likely to be due to both species difference and age differences between the two groups. It is known that younger fish are more reliant on aquatic respiration and this is correlated with the development and maturation of the supra-branchial organs (Moussa, 1957). Moussa (1957) found that dependence on aerial respiration increased with increasing body size. In the present study the smaller animal survived more than twice as long as the larger one. The results of the air-breathing frequency experiments suggest that the ventilation of the supra-branchial organs for an individual is variable (Table 3:2; Fig. 3:4, 3:5). The variability in the intervals between air-breaths and the fact that on acute exposure to hypoxia aerial respiration rate is significantly increased whereas air breathing frequency remains unchanged (Table 3:2, 3:3) suggest that the supra-branchial organs can be inflated according to need. The intervals between air-breathing are, thus, not a reliable method of assessing air-breathing effort.

Clarias mossambicus have very low concentrations of glycogen in the liver and slow muscle (Johnston & Bernard, 1983). Levels of 180 ± 63.6 and 65 ± 7.6 mg / 100g wet wt. tissue have been measured for

liver and slow muscle respectively (Johnston & Bernard, 1983). These values would indicate only a limited capacity for sustained anaerobiosis, as, generally species with high anaerobic capabilities have large liver glycogen stores in excess of 500 μmoles/g. wet wt. tissue (van den Thillart et al., 1976; Smith & Heath, 1980). Goldfish have liver glycogen concentrations of around 750 μm/g wet wt. tissue which are more than halved after 12 hrs complete anoxia (van den Thillart et al., 1976). It seems likely therefore, that Clarias have evolved air-breathing organs as an alternative rather than a complimentary strategy to anaerobic metabolism as a means of surviving in oxygen deficient water.

Few studies have been made of the effects of hypoxia acclimation on the respiration of air breathing fishes (Gee, 1980; Weber et al., 1979; Graham & Baird,1982). As was mentioned previously hypoxia acclimation in fish entirely dependent on aquatic respiration usually causes a compensatory increase in oxygen consumption due to an increased oxygen extraction capacity (Hughes, 1973; Wood & Johansen, 1972; Lomholt & Johansen, 1978; van den Thillart, 1982). Total respiration in Clarias mossambicus also increases after a period of acclimation (Table 3:3; Fig. 3:2b). An increase is observed in the aerial phase but is evident to a much greater extent (164% increase) in the aquatic phase (Table 3:3; Fig. 3:2b). This would tend to indicate that hypoxia cannot be completely compensated for by air-breathing but that the stimulus is sufficient to promote changes in factors such as circulation, ventilation and/or blood O_2 carrying capacity so increasing oxygen uptake in the aquatic phase. Further work is required to establish which of these factors are important in Clarias mossambicus but Fish (1956) has reported that the oxygen-dissociation curve of Clarias is very steep and relatively insensitive to CO_2. This property would favour loading of oxygen at the gills despite high concentrations of CO_2 accumulating in the supra-branchial organs between air-breaths. Increases in blood oxygen

affinity would increase oxygen uptake during air-breathing as well as in the gills.

The armoured loricariid catfishes, Ancistrus chagresi and Hypostomus plecostomus are facultative air-breathers which utilize their stomachs and gastrointestinal tracts as accessory air breathing organs. In a study of these species Graham and Baird (1982) found that the frequency of air-breathing was reduced after 14-21 days acclimation to hypoxia, however the threshold oxygen tension at which air breathing begun was unchanged. Weber et al. (1979) have demonstrated for the same species that oxygen affinity is increased by hypoxia acclimation due to decreases in both GTP and ATP concentrations in the erythrocytes. These results suggest that, similarly to Clarias mossambicus, despite air-breathing, internal oxygen tensions are low enough to induce physiological changes, in this case in the erythrocyte NTP concentrations, thus increasing blood O_2 affinity.

TABLE 3:1

	Oxygen tension	
	$P_{O_2} \approx$ 17.6 KPa	$P_{O_2} \approx$ 1.5 KPa
Fish acclimated to aerated water ($P_{O_2} \approx$ 17.6 KPa) Measurements made using flow through respirometers.	39.0 $\pm$ 4.6	18.7 $\pm$ 4.7
Fish acclimated to aerated water ($P_{O_2} \approx$ 17.6 KPa) Measurements made using closed respirometers.	32.7 $\pm$ 0.5	10.8 $\pm$ 0.8
Fish acclimated to hypoxic water ($P_{O_2} \approx$ 1.5 KPa) Measurements made using closed respirometers.	-	15.6 $\pm$ 1.7

Routine oxygen consumption (mls O_2 / Kg bodyweight / h) of tench, Tinca tinca acclimated to either hypoxic or aerated water.

TABLE 3:2

Air-breathing frequency of African catfish (Clarias mossambicus).

Condition	No. of fish	Hours observation	Interval between air-breaths (min) (Mean ± S.E.)	Range
Fish acclimated to aerated water $PO_2 \approx$ 15.1 KPa	8	3.3	9.5 ± 0.8	1.4 - 30.6
Initial response (< 36h) to hypoxic water $PO_2 \approx$ 2.4 KPa	8	5.6	9.8 + 1.3	1.3 - 32.6
Fish acclimated to hypoxia ($PO_2 \approx$ 2.4 KPa) for 11-13 days	8	3.7	7.4 + 0.8	2.3 - 18.4

TABLE 3:3

Routine respiration rates of Clarias mossambicus acclimated for 27 days to either aerated ($PO_2 \approx 15.3$ KPa) or hypoxic water ($PO_2 \approx 2.4$ KPa) at 20°C. Values represent mean ± S.E.

PO_2 water (KPa)	No. of fish	Aquatic respiration ($mls\ O_2.Kg^{-1}\ hr^{-1}$)	Aerial Respiration ($mls\ O_2.Kg^{-1}\ hr^{-1}$)	Total Respiration ($mls\ O_2.Kg^{-1}\ hr^{-1}$)	% Air breathing
Fish acclimated to aerated water ($PO_2 \approx 15.3$ KPa)					
15.1	7	64.6 ± 6.5	21.1 ± 1.9	85.7 ± 6.8	24.6 ± 1.9
3.5 - 2.0	7	10.4 ± 1.4	35.9 ± 4.0	46.3 ± 4.9	77.5 ± 2.4
Fish acclimated to hypoxic water ($PO_2 \approx 2.4$ KPa)					
3.5 - 2.0	8	27.5 + 6.3	40.3 + 5.3	67.8 ± 7.3	59.4 ± 5.3

Figure 3:1

The oxygen tension dependent oxygen consumption by maximally active or inactive fish and the hypothetical influence of hypoxia acclimation. After Fry, 1947.

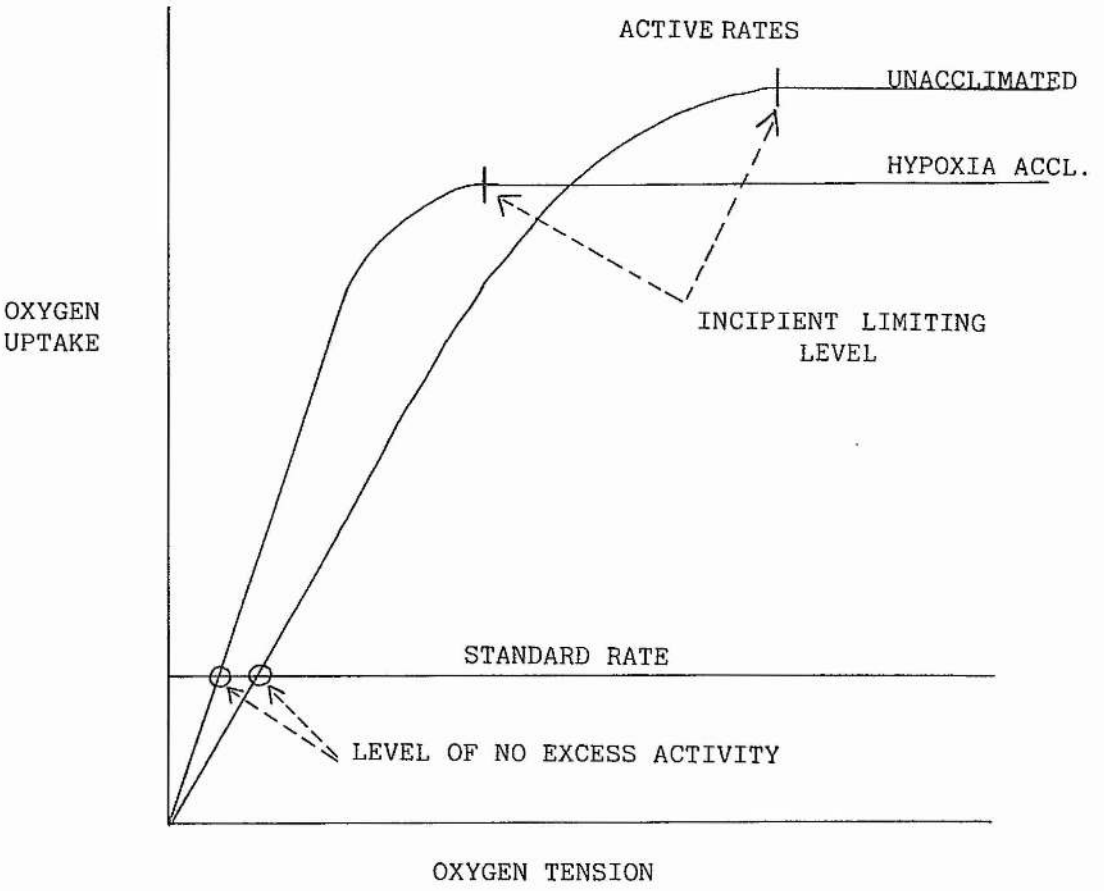
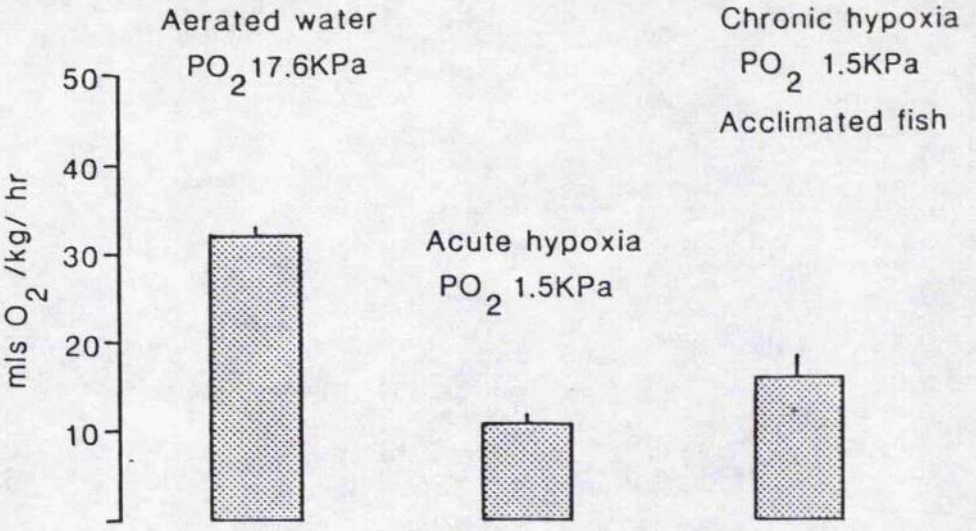

ACTIVE RATES
UNACCLIMATED
HYPOXIA ACCL.
OXYGEN
UPTAKE
INCIPIENT LIMITING
LEVEL
STANDARD RATE
LEVEL OF NO EXCESS ACTIVITY
OXYGEN TENSION

Figure 3:2

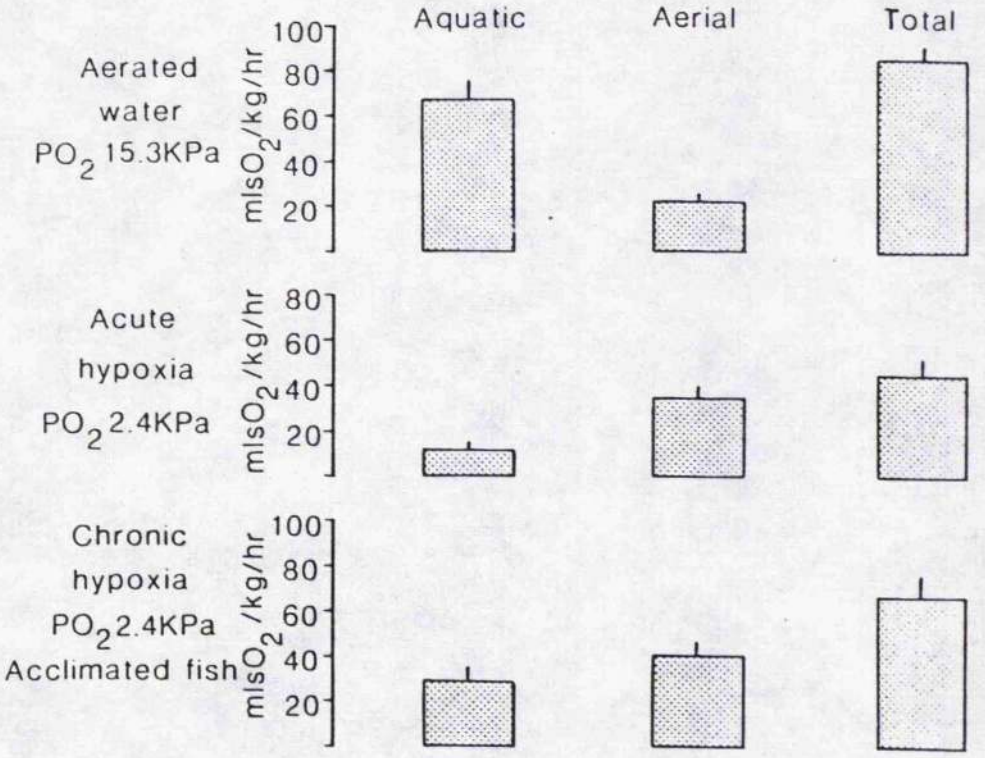
(a) Histograms showing the effect of acclimation to chronic hypoxia for six weeks on the respiration of the tench, Tinca tinca.

(b) Histograms showing the effect of acclimation to chronic hypoxia for 27 days on the aquatic, aerial and total respiration of the catfish, Clarias mossambicus.



Effects of acclimation to hypoxia on respiration in the tench (Tinca tinca)(at 15°C)

a



Effects of acclimation to hypoxia on respiration in the African catfish, Clarias mossambicus (at 20°C)

b

Fig 3.2

Figure 3:3

Graph showing the effects of progressive hypoxia on routine oxygen consumption (mls O_2 / Kg / h) of Crucian carp acclimated to either aerated (normoxia) (closed circles) or hypoxic (open circles) water.

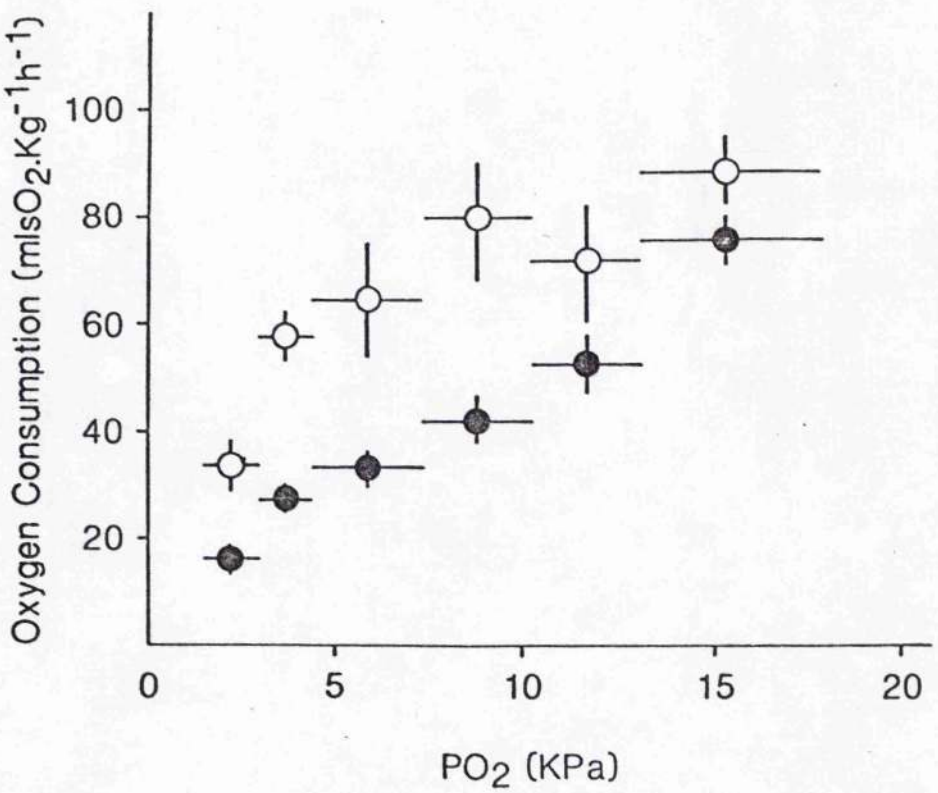

Oxygen Consumption (mlsO2.Kg-1h-1)
100
80
60
40
20
0
5
10
15
20
PO2 (KPa)

Figure 3:4

Frequency histograms of the intervals between air breaths for 8 individual catfish, Clarias mossambicus, acclimated to aerated water ($P_{O_2} \approx 15.3$ KPa). Fish were observed for a total of 3.3 h. Each bar represents a two minute time interval.

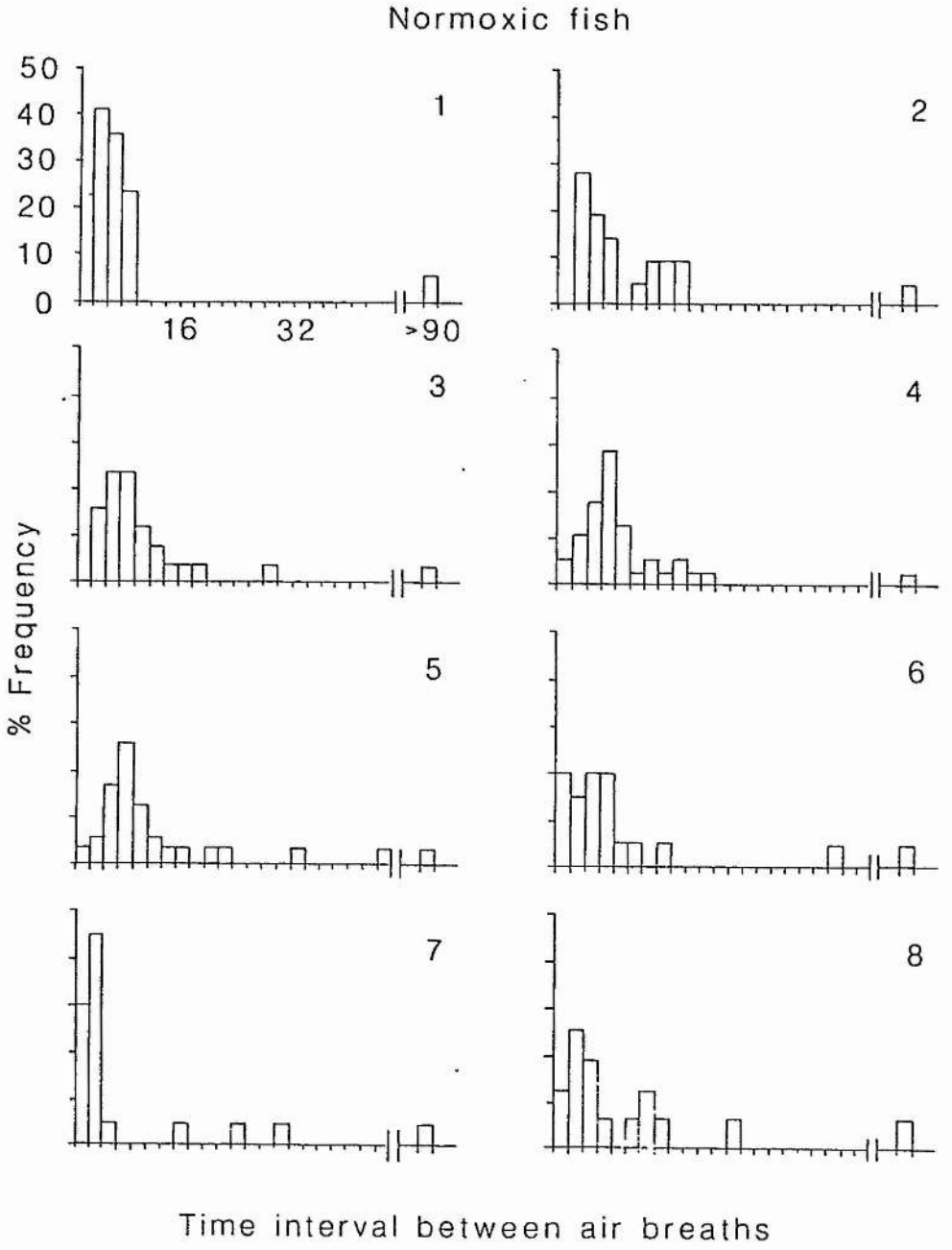

Normoxic fish
50
40
30
20
10
0
16
32
>90
1
2
3
4
5
6
7
8
% Frequency
Time interval between air breaths

Fig 3.1a

Figure 3:5

Frequency histograms of the intervals between air-breaths for 8 catfish, Clarias mossambicus, acclimated to hypoxic water ($P_{O_2} \approx 2.4$ KPa). Fish were observed for a total of 3.7 h. Each bar represents a 2 minute time interval.

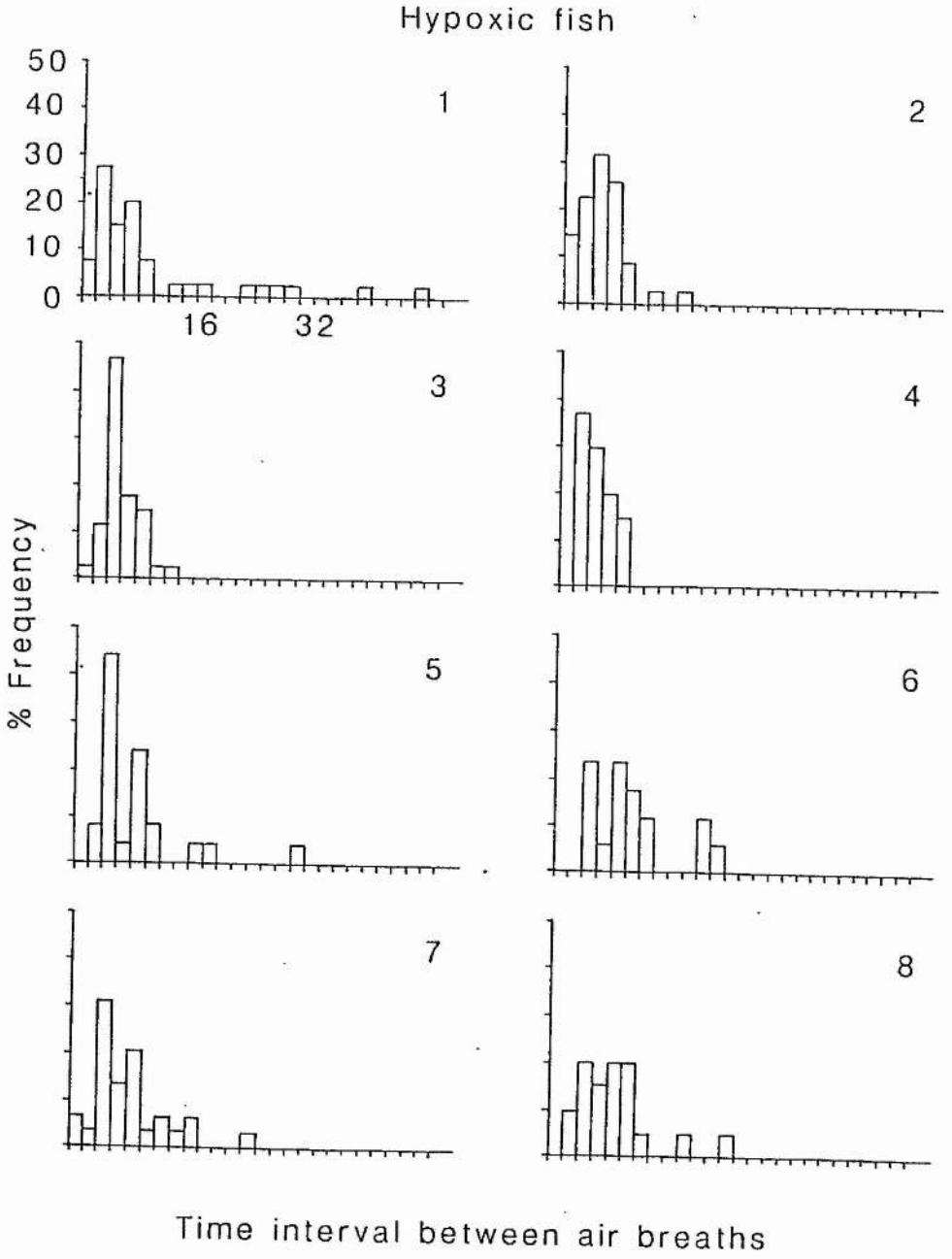

Hypoxic fish
50
40
30
20
10
0
16
32
1
2
3
4
5
6
7
8
% Frequency
Time interval between air breaths

Fig 3.3.

CHAPTER 4

FIBRE TYPES AND INNERVATION

Introduction

Amongst the teleost fish there is little variation in the types of myotomal muscle fibre present and their location (see Chapter 1). All species possess the physiologically distinct 'red' and 'white' fibres and many the intermediate or 'pink' type but the appearance of minor fibre groups is species specific. It was therefore necessary to establish the fibre types present and their precise location for the species under study by means of histochemical staining of frozen sections for glycogen, SDHase activity and myofibrillar ATPase activity which enabled a classification of fibre types to be made. Of importance also is the innervation pattern of the fibre types. Not only does this give a clue towards the classification of the fibres but in species where the taxonomic classification has not been established it provides a useful character in elucidating this (Bone & Ono, 1982), however this is well established for the species under study.

Materials and Methods

The histochemistry and innervation studies of the myotomal muscalature were carried out on the tench and catfish. Histochemistry and innervation of the Crucian carp muscalature have already been examined by Johnston (1974) and Bone (1982) and so will only be reviewed here.

The tench, Tinca tinca L. were obtained during October 1980 from Stanbridge trout fisheries, Rochford, England. Four fish of mean length 16.0 ± 0.7 cm were used.

The histochemistry of the catfish, Clarias mossambicus, Richter,

was performed on a single 56g specimin, kindly donated by the Institute of Aquaculture, Stirling University, Scotland. Methods were as detailed in Chapter 2.

Results

The tench, Tinca tinca L.

The myotomal muscalature of the tench is divisible into three main fibre types on the basis of histochemical criteria. On the basis of colour, the fibres are red, pink and white but recent studies have shown that histochemical data on myofibrillar ATPase activity and aerobic capacity (Johnston et al., 1974; Bone, 1978a;; Johnston, 1982 b), can be correlated with data on the contractile properties and speeds of shortening of muscle fibres (Johnston, 1980 a, b, 1981; Altringham & Johnston, 1981 b), allowing the red, pink and white fibres to be classified as slow, fast aerobic and fast glycolytic, respectively.

The slow fibres lie just underneath the skin forming a wedge in the lateral line nerve region. Histochemically they are distinguishable from the other fibre types by their alkaline labile myofibrillar ATPase activity, inactivation occurring after 2 minutes preincubation at pH10.4. Glycogen levels are high and staining for succinic dehydrogenase activity, a marker for oxidative pathways is intense (Fig. 4:1 a-g).

Beneath the slow fibres is a zone of pink or fast aerobic type fibres. They have high myofibrillar ATPase activity, intermediate SDHase activity and intermediate glycogen levels, (Fig. 4:1 a-g), and are distinguishable from all other fibre types by the stability of their Ca^{2+} activated myofibrillar ATPase activity to alkaline pre-incubation (Fig. 4:1 a, c). They retain their ATPase activity (Fig. 4:1 a, c) following up to 20 minutes pre-incubation at pH 10.4.

The main bulk of the body muscatature consists of the white or fast

glycolytic fibres. These fibres are characterised by their high, alkaline labile myofibrillar ATPase activity, low glycogen levels and low staining intensity for SDHase activity (Fig. 4:1 a, b, d, e, f).

The boundary between the fast aerobic and fast glycolytic fibres is not distinct (Fig. 4:1 c, e). Staining for Ca^{2+} activated ATPase activity after alkaline pre-incubation shows a mosaic appearance of heavily stained, fast aerobic and lightly stained fast glycolytic fibres around the boundary region (Fig. 4:1 c, e).

Towards the lateral-line nerve there was a 12-cell deep layer of fibres of very small diameter which stained relatively weakly for succinic dehydrogenase activity (Fig. 2:4).

The innervation of the muscle fibres in the tench is typical of the advanced teleosts. All fibre types are multiply innervated by a diffuse network of nerves (Fig. 4:2 a-g). Each slow fibre is served by several different axons which branch giving rise to a large number of endplates on the same and adjacent fibres (Fig. 4:2a). Fast glycolytic fibres also receive a large number of endplates (8-20 per fibre) (Fig. 4:2 b, c, g), although the structure of the endplates is more discrete and varies considerably even along a single fibre (Fig. 4:2 c-g). Generally individual endplates are comprised of a series of small loops and terminal buttons (Fig. 4:2 d-g). A small degree of multi-terminal innervation is present (Fig. 4:2 c, e), however the majority of endplates appear to be derived from different axons (Fig. 4:2 b).

The catfish, Clarias mossambicus, Richter.

In common with the carp and tench the axial muscalature of the catfish is divisable into three main fibre groups on the basis of histochemical staining techniques (Fig. 4:3 a-f). The slow or red fibres form a superficial wedge beneath the skin in the lateral line region

and show no staining for myofibrillar ATPase after 6 min preincubation at pH 10.4 (Fig. 4:3 b, e). Staining for glycogen and SDHase activity reveals a relatively high intensity reaction (Fig. 4:3 a, c, f). After 12 mins pre-incubation at pH 10.4 the m.ATPase of the fast glycolytic (white) fibres is inactivated (Fig 4:3 b, e). These fibres show only a weak staining for glycogen and SDHase activity (Fig. 4:3 a, c, f) and have a generally larger diameter than the slow fibres.

Between the slow and fast glycolytic fibre layers lies a population of fibres with a wide range of fibre sizes and a staining for glycogen and SDHase activity intermediate to the slow and fast groups. Their m.ATPase is inactivated only after 15 min pre-incubation at pH 10.4 and they are classified as the fast aerobic or pink fibre type (Fig. 4:3 a-f).

The slow fibres in the catfish have a complex distributed innervation with around 10-15 endplates per fibre (Fig. 4:2 h). Each fibre receives branches from several different axons.

The fast glycolytic fibres have a type of innervation which is distinct from that of the slow fibres and is characteristic of the more phylogenetically primitive groups of teleosts. Each fast glycolytic fibre is innervated at one myoseptal end by an "en plaque" type end plate consisting of numerous button-like vesicles (Fig. 4:2 i).

Discussion

The fibre types found in the Crucian carp, Carassius carassius, are very similar to those of the tench and catfish and typical of the advanced teleosts. Johnston et al. (1974) have shown three main fibre types, distinguishable by their responses to alkaline pre-incubation for differing time periods before staining for myofibrillar ATPase activity, and classifiable as slow, fast oxidative and fast glycolytic

on the basis of measurements of their relative contraction speeds and metabolic characteristics (Altringham & Johnston, 1982b; Johnston, 1983).

Pre-incubation at pH 10.4 for up to 3 mins before staining for myofibrillar ATPase activity leads to an inactivation of the slow (red) fibre m.ATPase and shows dark staining in only the fast aerobic (pink) and fast glycolytic (white) fibres (Johnston et al., 1974). Pre-incubation of 15 mins gives inactivation of both slow and fast muscle m.ATPase with only the fast aerobic muscle fibres staining heavily. In common with the tench the carp muscle fibres show a progressive decrease in staining intensity for glycogen and SDHase activity in the order slow > fast aerobic > fast glycolytic fibres (Johnston et al., 1974).

Innervation of the muscle fibres in the Crucian carp is again typical of the advanced teleosts. All fibres are multiply innervated by a diffuse network of nerves stemming from branches of the spinal nerves which run into the myosepta. Each fibre receives numerous nerve terminals with "en grappe" type endplates (Bone, 1968).

The types of fibre present and their location as determined by histochemical staining for m.ATPase activity, SDHase activity and glycogen in the three species under study show a close degree of similarity to the pattern found in the majority of teleosts (see Chapter 1). In all three species the slow fibres are located in a superficial wedge in the lateral line area. Beneath the slow muscle lies a region of fast aerobic muscle. This type of muscle has been shown to be present in many species of fish (Johnston, 1980). The rest, and by far the bulk, of the myotomal muscalature is made up of fast glycolytic muscle.

The establishment of the presence of homogenous fibre groups in all three species under study allows directly comparable samples to be taken for ultrastructural investigation.

The type of innervation observed in the three species is similar with the exception of the white muscle of the catfish. The slow muscle

fibres are multiply innervated and in the cases of the tench and Crucian carp so are the fast glycolytic fibres. This type of innervation is usually consistent with species which recruit their fast glycolytic fibres over a wide range of swimming speeds (Hudson, 1973; Johnston et al., 1977; Bone et al., 1978; Johnston & Moon, 1980 a, b). Activity is detectable during moderate cruising (0.5 - 2.0 body lengths / s) and burst swimming speeds in the Brook trout, Salvelinus fontinalis and the carp, Cyprinus carpio (Johnston & Moon, 1980a), both of which have multiple innervation of the white fibres, however, in some species with multiply innervated white fibres, such as the striped bass, Morone saxitilis and the bluefish, Pomatomus sultatrix, very much higher swimming speeds are reached before the white fibres are recruited (> 4 body lengths / s) (Freadman, 1979). This variation in fibre recruitment may reflect differences in either the number of neuromuscular endplates per fibre and/or the degree of motor axon branching or multiterminal innervation. The cod, Gadus morhua, has a similar number of endplates per fast fibre to the tench and both show a small degree of branching of axons to form two or more endplates on the same fibre (Altringham & Johnston, 1981a). This is contradictory to Hudson's theory (1969) that each fast muscle endplate is derived from a separate motor axon. In the cod each fast axon usually branches terminally to innervate two to six adjacent fibres (Altringham & Johnston, 1981a), whereas, in the tench terminal axon branching is evident to a much lower degree (Fig. 4:2 e)

The innervation of the fast glycolytic muscle in the catfish is very different from that of the tench and Crucian carp. In the catfish the muscle fibres are innervated only at their myoseptal ends so that innervation is focal and terminal. This mode of innervation is rare in teleosts (Barets, 1961; Bone, 1964), but is universal in non-teleost groups (Bone & Ono, 1982). It has been proposed that the focal/terminal

innervation pattern is the primitive condition from which the distributed pattern is derived (Bone & Ono, 1982). Notable differences have been observed in the locomotor activities between the fishes where the white muscle is terminally innervated and where it is innervated in the distributed manner. Electromyographical records from species where the white muscle fibres are terminally innervated show that these are active during bursts of rapid swimming and are rapidly exhausted. For example in the Pacific herring and the dogfish activity is detectable only from the red fibres during sustainable swimming speeds. The white fibres are recruited at higher speeds (> 5 lengths / s) and the fish fatigues within a few minutes (Bone, 1966; Bone et al., 1978). The catfish, Clarias mossambicus, is a relatively sedentary species spending much of its time in the bottom mud of rivers and lakes so it seems likely that it will have little need to sustain swimming speeds for any length of time which may be the reason for the retention of the primitive innervation pattern in this species.

Figure 4:1

Histochemistry of tench, Tinca tinca, myotomal muscle

(a) Section stained for myofibrillar ATPase following alkaline pre-incubation for 2 min at pH 10.4. Note the inactivation of slow fibres (S) from the lateral line triangle and fast glycolytic fibres (FG). Only aerobic fast fibres (FA) show staining for ATPase activity following alkaline pre-incubation. In addition to a discrete zone of FA fibres, note region containing a mixture of both aerobic and glycolytic fibres giving muscle a characteristic mosaic appearance.

(b) Section stained for myofibrillar ATPase activity without prior alkaline pre-incubation. Note the greater density of staining of fast aerobic (FA) than slow fibres (S).

(c) Section stained for myofibrillar ATPase following 3 min alkaline pre-incubation showing the interface between discrete areas of fast aerobic (FA) and fast glycolytic (FG) fibres.

(d) Transverse section of superficial slow fibres (S) adjacent to the skin stained for succinic-dehydrogenase activity.

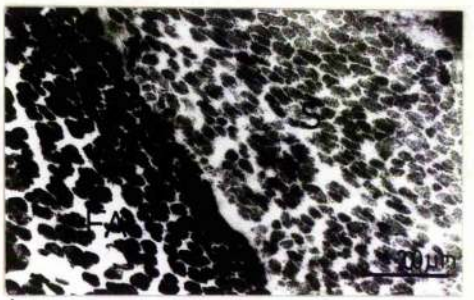
(e) Transverse section incubated for succinic-dehydrogenase activity showing heavily stained slow (S) intermediately stained fast aerobic (FA) and relatively poorly stained fast glycolytic fibres (FG). Note the wide range of sizes of fast glycolytic fibres overlapping that of the slow fibre population (also in (d)).

(f) Transverse section showing slow (S) and fast aerobic (FA) fibres stained for glycogen by the PAS method.

(g) Semithin (1μm) Araldite embedded section of slow muscle stained with p-phenylene diamine. Note the presence of darker-staining areas corresponding to subsarcolemmal and intermyofibrillar mitochondria and extracellular lipid deposits (L).

(h) Semithin (1μm) section of slow fibres stained with toluidine blue. Note the relatively abundant capillary supply (C), extracellular lipid (L) and the variable structure of the individual slow fibres.

a



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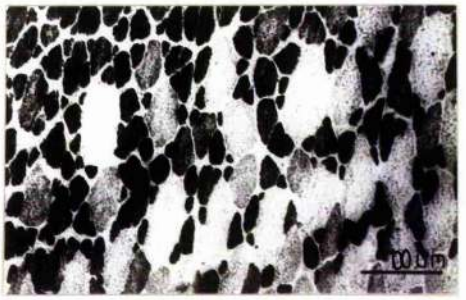
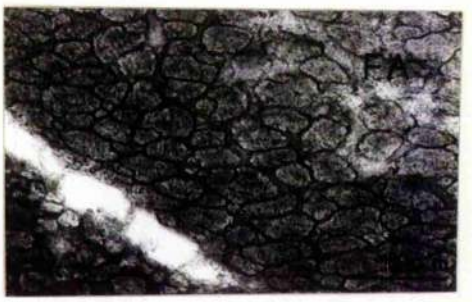


c

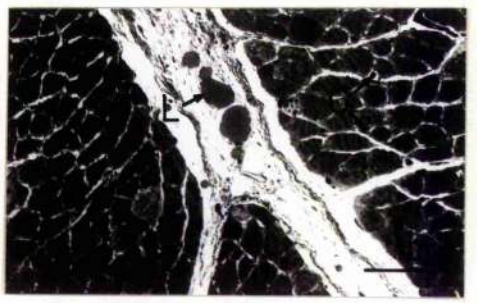
d

e



f

g



h

Figure 4:2

Teased fibre bundles of tench (a-g) and catfish (h, i) myotomal muscle stained for acetylcholinesterase activity to show the distribution of endplates and pre-terminal axons.

(a) Slow fibres from the tench. The myoseptal insertion is visible at bottom left. Note the extensive innervation with the large number of endplates per fibre.

(b) Fast fibres from the tench. These have an extensive innervation and a large number of endplates per fibre however the endplates are more discrete than those of slow fibres (a).

(c) Fast fibres from the tench. Note the branching of preterminal axons.

(d) Fast fibres from the tench. Note the structure of individual endplates consisting of several loops and terminal buttons.

(e) Fast fibres from the tench. Single axon (B) branches to give endplates on three different fibres. Note only two branches are visible in the plane of focus. Fibre A appears to receive two endplates from the same axon.

(f) A segment of a single fast muscle fibre from the tench showing four distinct endplates of variable form.

(g) A bundle of five fast fibres from the tench showing the variable structure and number of endplates.

(h) Slow fibres from the catfish. Note the diffuse endplates distributed along the lengths of the fibres.

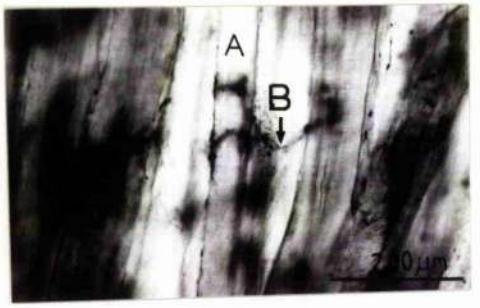
(i) Fast fibres from the catfish. "Enplaque type" endings are visible at the insertion of fibre with the myoseptum (MS).

a

b

c

d



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g

h

i

Figure 4:3

Histochemistry of the catfish, Clarias mossambicus, myotomal muscle.

(a) Section stained for succinic dehydrogenase activity showing slow (S), fast aerobic (FA) and fast glycolytic (FG) muscle fibre zones.

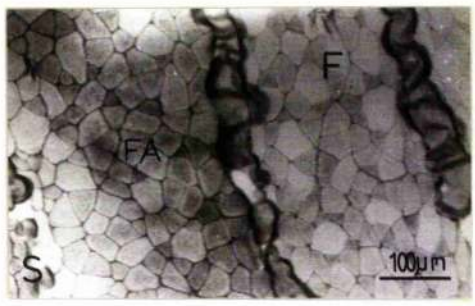
(b) Red and intermediate muscle zones stained for myofibrillar ATPase activity following a 6 minute pre-incubation at pH 10.4. S, slow fibres; F, fast fibres.

(c) Section stained for succinic dehydrogenase activity showing the boundary between the slow (S), fast aerobic (FA) and fast glycolytic (FG) fibre zones.

(d) Semi-thin (1µm) section stained for toluidine blue showing muscle fibres from a catfish acclimated to hypoxic water. S, slow fibres; FA, fast aerobic fibres; FG, fast glycolytic fibres; C, capillaries.

(e) Intermediate muscle zone stained for myofibrillar ATPase activity following 6 minutes pre-incubation at pH 10.4 Note 'mosaic' appearance at boundary with red fibres.

(f) Section stained for succinic dehydrogenase activity showing the boundary between the slow (S) fast (F) fibre zones.



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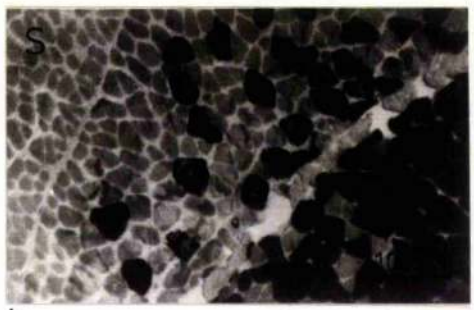

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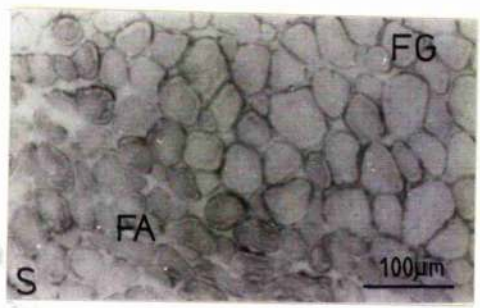

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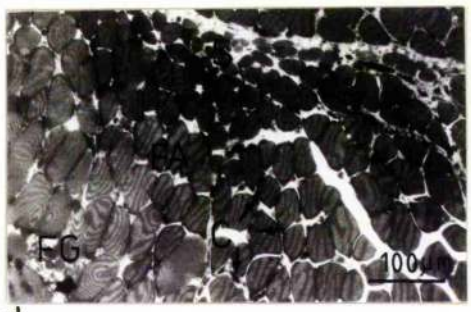

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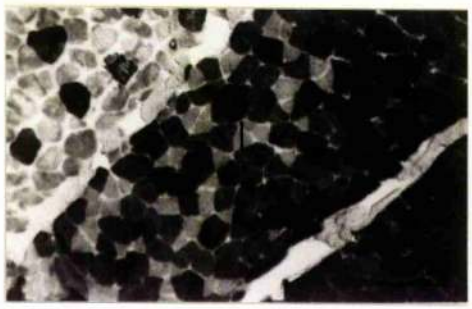

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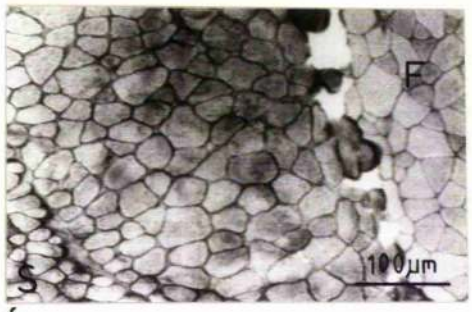

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CHAPTER 5

THE EFFECTS OF ACCLIMATION TO HYPOXIA ON THE ULTRASTRUCTURE OF TENCH, (TINCA TINCA L.) MYOTOMAL MUSCLE FIBRES

It has been established in Chapters 3 and 4 that the myotomal musculature of the tench is composed of three main fibre types arranged in the pattern typical of the majority of teleosts and that acclimation to hypoxic conditions results in adaptation of the animal allowing it to extract more oxygen from its hypoxic environment. This chapter investigates the ultrastructure of the slow and fast glycolytic fibres in the tench, Tinca, tinca, L, and reports the changes brought about in them by acclimation to hypoxia.

Although there have been a number of quantitative ultrastructural studies on the muscles of various fish species (see Chapter 1), few authors have made comparative studies of muscle fibres of fish acclimated to various variable environmental conditons (Johnston, 1982a; Patterson & Goldspink, 1973; Johnston & Maitland, 1980ab; Beardall & Johnston, 1983). In a study of the Crucian carp, Carassius carassius, Johnston and Maitland (1980) found large increases in both the number of mitochondria and the fractional volume occupied by them in all fibre types following acclimation to low temperatures and the fractional volume of fibre occupied by myofibrils was decreased slightly. Penney, Johnston and Goldspink (1979) found an increase in the surface area of the sarcoplasmic reticulum relative to the myofibril volume in cold acclimated goldfish. It seems likely, therefore, that exposure to hypoxia for a period of time will induce such changes in the ultrastructure of fish muscle fibres.

Fish slow fibres are all multiply innervated and characteristically have mitochondrial volume fractions in the range 20 - 45% with a relatively highly developed sarcotubular system (See Chapter 1; Bone, 1978b; Johnston, 1982a; McArdle & Johnston, 1981), however the ultrastructural characteristics of the fast glycolytic fibres are dependant on their type of innervation. In the elasmobranchs and other groups with the terminal type innervation, mitochondrial volumes in the fast fibres are typically less than 1% (Kryvi, 1977). In contrast the multiply innervated fast fibres of the teleosts typically have mitochondrial fractions in the range 2 - 9% (Johnston, 1981a; Johnston & Moon, 1981).

To my knowledge there are no other studies on the effects of acclimation to hypoxia on fish muscle ultrastructure.

Materials and Methods

Sixteen tench (Tinca tinca L.), of mean standard length 16 cm were divided into two groups and acclimated to either aerated or hypoxic water as described in Chapter 2. Ultrastructural and quantitative methods are detailed in Chapter 2.

Results

As was stated in Chapter 4 the myotomal muscle of the tench is composed of three main fibre types, slow, fast aerobic and fast glycolytic. Since it was not possible to dissect reliably homogenous samples of the fast aerobic (pink) fibres, they were not included in the quantitative analyses although direct observations showed them to have mitochondrial and myofibrillar volume fractions intermediate between slow and fast glycolytic fibres (Fig. 5:1 d). Thus, this study concentrates only on the ultrastructure of the

slow (red) and fast glycolytic (white) fibres.

In fish acclimated to aerated water, fibres from the slow muscle zone show a marked heterogeneity in their fine structure particularly with respect to the size of the subsarcolemmal space (Figs. 2:4b, 5:1c, 5:2a, 5:3a, b, c). The size of the subsarcolemmal space is apparently dependant upon the depth of the fibre within the slow muscle zone, the size of the subsarcolemmal space decreasing towards the inner boundary of the slow muscle zone (Fig. 2:4b). The proportion of fibres with large subsarcolemmal space and the depth of this zone was variable between individual fish therefore the quantitative ultrastructural analyses was based on a random sampling technique.

The percentage volume occupied by mitochondria varied considerably among slow fibres, the mean being around 23%, and it was not related to the fibre cross-sectional area (Fig. 5:4A). Subsarcolemmal mitochondria occupied between 3 and 40% the mean value being 18.5% (Table 5:1; Fig. 5:2a). Intermyofibrillar mitochondria occupied a substantially smaller compartment ranging from 0 to 13% with a mean of 4.4% (Table 5:1; Fig. 5:2a). Thus, on average there were four to five times as many subsarcolemmal as intermyofibrillar mitochondria both in terms of numbers and fractional volumes (Table 5:1).

Myofibrils accounted for 20 - 70% of slow fibre volume, the mean being 43% (Table 5:1, Fig. 5:8). The T-tubules were located at the level of the Z-disc in both fast and slow fibres (Fig. 5:5 a, b) and the M-line is conspicuous (Fig. 5:5 a, b). The fraction of slow fibre myofibril volume occupied by the sarcoplasmic reticulum was 4.4% and by T-system 0.29% (Table 5:1; Fig. 5:5a). This is relatively low compared to those of trout (Nag, 1972) and sharks (Kryvi, 1977).

The composition of the fast glycolytic fibres is quite different from that of the slow fibres. Fibre diameter ranges from 10 - 160 μm, the smaller of these probably representing growing fibres. The smaller fibres with cross-sectional areas less than 750 μm^2 tend to have somewhat higher volume fractions of mitochondria (Fig. 5:4B). Amongst the fast fibres mitochondrial volume fraction ranged from 0 to 12% (Fig. 5:2b; 5:3b), the majority of these being located in the subsarcolemmal zone (Table 5:2; Fig. 5:1f). The smaller, probably growing, fibres often contained extensive glycogen deposits (Fig. 5:1e). Myofibrils occupied a mean fractional volume of 73% (Table 5:2), the range being 50 - 90% (Fig. 5:8b). Sarcoplasmic reticulum composed around 3.2% of myofibril volume and T-system 0.4% (Table 5:2; Fig. 5:5b).

The layer of small diameter fibres found near the lateral line nerve (Fig. 2:4b) and characterised histochemically by their relatively weak staining for succinic dehydrogenase activity (see Chapter 4) are ultrastructurally distinct from the other fibre types and could possibly represent a growth stage of the slow fibres. The subsarcolemmal space in these fibres is large and there are few intermyofibrillar mitochondria. The outer myofibrils are typically elongated and arranged like the spokes of a wheel round a few central polygonal ones.

The major effect of acclimation to hypoxia on the ultrastructure of the slow and fast glycolytic fibres of the tench is on the fractional volume occupied by mitochondria. In both fibre types the percentage decreased significantly, falling in the slow fibres from 23% to 15% (Table 5:1) and in the fast glycolytic fibres from 4.5% to 1.8% (Table 5:2), decreases in fractional volume of 35% and

60% respectively (Table 5:1, 5:2). A proportionally greater decrease in volume fraction occurred in the intermyofibrillar mitochondria. This fell by 27% in the slow and 93% in the fast glycolytic fibres compared to falls of 25% and 53% respectively in the subsarcolemmal mitochondria (Tables 5:1, 5:2; Figs. 5:1c, 5:2, 5:3, 5:6a, b, c).

The actual numbers of mitochondria per fibre cross-section decreased more than would be expected from a consideration of the volume of the mitochondrial compartment (Tables 5:1, 5:2). This would indicate an increase in average mitochondrial size with acclimation to hypoxia. In a proportion ($\approx$ 20%) of fibres from hypoxia acclimated fish mitochondria were found, amongst normal mitochondria, which appeared swollen with large inner spaces and few, simple cristae (Fig. 5:7 c, d). Mitochondria from normoxic acclimated fish are shown in Fig. 5:7 a, b.

The fractional volume occupied by myofibrils was also affected to a lesser, but none the less significant, level by acclimation to hypoxia. Increases were observed in both fibre types, rising from 43% to 56% in the slow and 73% to 83% in the fast fibres (Tables 5:1, 5:2; Fig. 5:8 a, b), a net increase of 28% in slow and 13% in fast fibres.

Acclimation to reduced oxygen levels was also associated with a small but significant ($P < 0.05$) decrease in the fractional volume of myofibrils occupied by sarcoplasmic reticulum in the slow fibres. This dropped by 25%, however, in the fast fibres there was a massive increase of 103% (rising from 3.2% to 6.5%) (Tables 5:1, 5:2). The fractional volume occupied by T-system dropped slightly from 0.29% to 0.24% in the slow fibres and from 0.4% to 0.26% in the fast (Tables 5:1, 5:2).

Growth was still occurring in both groups as actively differentiating fibres could be seen (Fig. 5:7e). These fibres

were characterised by large end regions containing extensive endoplasmic reticulum and dense glycogen deposits (Fig. 5:7e).

Discussion

The ultrastructure of the slow and fast glycolytic fibres of the tench, both in transverse and longitudinal section, is broadly similar to most of the other teleosts examined (Akster, 1981; Johnston, 1981a,b; 1982a; Patterson & Goldspink, 1972; Walesby & Johnston, 1980; Johnston & Maitland, 1980). Both fibre types possess the distinct M-line and well developed triads at the level of the Z-disc (Kilarski, 1967; Nishihara, 1967; Patterson & Goldspink, 1972; Hulbert & Moon, 1978). Only in fish muscle and the tail muscle of urodeles is the M-line found in slow fibres (Kilarski, 1967; Nishihara, 1967; Totland, 1976). Generally slow fibres are found to have less extensive membrane systems than fast (Page, 1968b; Hess, 1970; Franzini-Armstrong, 1973; Nag, 1972; Kilarski, 1973; Patterson & Goldspink, 1972; Kryvi, 1972). In the rainbow trout SR occupies 5.1% of myofibril volume in slow fibres and 13.7% in fast, however, in the tench the volumes for both fibre types are not significantly different (3 - 4%) and relatively low.

The pattern of ribbon like peripheral myofibrils with polygonal internal fibrils characteristic of the teleosts is present in the fast glycolytic fibres in the tench.

This study shows conclusively that the fractional volumes occupied by various cellular components are altered after acclimation to hypoxia.

The major factor influenced by hypoxia is the fractional volume occupied by mitochondria. Large decreases are observed in both slow (35%) and fast fibres (60%). Müller (1976) has

proposed that subsarcolemmal and intermyofibrillar mitochondria represent biochemically and functionally distinct populations. He suggests that the subsarcolemmal mitochondria mainly supply energy for active transport across the sarcolemma whilst the intermyofibrillar population supply the energy for contraction (Müller, 1976). It seems unlikely that such a simple division of labour holds for tench slow fibres since the relatively low fraction of intermyofibrillar mitochondria (4.4%) would almost certainly be insufficient to supply the energy required for sustained swimming (Table 5:1).

The significantly larger decrease in intermyofibrillar mitochondria as opposed to subsarcolemmal mitochondria may be attributable to their large distance from the source of oxygen and nutrients. Subsarcolemmal mitochondria are relatively close to capillaries and their metabolism will lead to a progressively decreasing gradient of oxygen and nutrients towards the centre of the fibre. Hypoxia will further decrease this gradient and this may lead to central mitochondria being starved of essential oxygen and nutrients resulting in their atrophy and phagocytosis. A proportion of slow fibres from hypoxic fish showed evidence of such degeneration (Fig. 5:7 c, d). Alternatively these mitochondria may be degenerating simply because there is no need for them. Normally, in their natural habitat, tench experience hypoxia during their winter hibernation phase. During hibernation, buried in the mud, the animals remain totally quiescent. If no movement is occurring then less mitochondria would be required to maintain the fibre, especially in the myofibrillar zone therefore some might be broken down. An examination of hibernating tench might reveal even lower levels of mitochondria than found in the hypoxia acclimated animals, however this is purely speculative.

The decrease in mitochondrial volume in hypoxia acclimated tench fibres was accompanied by a parallel decrease of around 33% in routine oxygen consumption (see Chapter 3), muscle cytochrome oxidase activities (Johnston & Bernard, 1982b) and in the number of capillaries per unit volume of muscle fibres (see Chapter 6). Phosphofructokinase activities were increased in the slow fibres as were glycogen levels in both fibre types, though only slightly (Johnston & Bernard, 1982b).

All these factors are in keeping with an enhanced capacity for glycolysis. During anoxia, glycogen in the liver and slow muscles is the principal energy source for metabolism (van den Thillart et al., 1976). Large glycogen stores in the liver and slow muscles are generally found in anoxia tolerant species (van den Thillart et al., 1976; Smith & Heath, 1980) and those of the tench are broadly similar to those of other anoxic tolerant species such as the goldfish (van den Thillart et al., 1980) and significantly higher than anoxia intolerant species such as the rainbow trout (Heath & Pritchard, 1965).

Although respiration in the tench is reduced after acclimation to hypoxia it seems likely that aerobic pathways are able to supply all the energy requirements of basal metabolism under these conditions, however the enhanced capacity for anaerobic energy production would become important when activity occurred. In fish acclimated to hypoxia anaerobic energy production would be necessary at lower levels of muscular activity than in fish acclimated to aerated water. The enhanced capacity for glycolysis may therefore reflect a reduced threshold for anaerobic metabolism during activity and/or an adaptation in anticipation of acute exposure to anoxia in fish acclimated to hypoxia.

Tench are very tolerant to hypoxia and can continue to live and grow at very low oxygen tensions indefinitely. Evidence of actively growing myofibrils has been found in tench acclimated to hypoxia (Fig. 5:7e).

Fish muscle is obviously very adaptable to environmental change. The major influence of hypoxia is on the mitochondrial volume of the fibres and it seems that this is closely related to the capillary supply to the muscle. This relationship is discussed in the next chapter both for the tench and for the Crucian carp and catfish.

Table 5:1

Effects of acclimation to hypoxic conditions on the fine structure of tench slow muscle fibres.

Parameter Mean ± S.E.	Environmental oxygen level $Po_2 \approx$ 21.0KPa	 $Po_2 \approx$ 1.7KPa
No. fibres analysed	62	90
Fibre area (μm^2)	345.85 ± 28.4	323.90 ± 23.8
Fibre perimeter (μm)	74.44 ± 3.77	100.82 ± 4.4
Fibre area occupied by subsarcolemmal zone (%)	44.9 ± 1.4	40.0 ± 1.2
No. subsarcolemmal mitochondria per fibre cross-section	100.3 ± 8.2	45.5 ± 3.1***
No. interfibrillar mitochondria per fibre	23.8 ± 2.6	6.0 ± 0.5***
Fractional volume of mitochondria (total) (%)	22.9 ± 1.1	15.0 ± 0.8**
Fractional volume of subsarcolemmal mitochondria (%)	18.5 ± 0.9	13.8 ± 0.7
Fractional volume of inter-fibrillar mitochondria (%)	4.4 ± 0.6	1.2 ± 0.2***
Fractional volume of myofibrils (%)	43.1 ± 1.2	56.1 ± 1.2**
Fractional volume of sarcoplasmic reticulum (%)*	4.4 ± 0.2	3.3 ± 0.2**
Fractional volume of T-system*	0.29 ± 0.02	0.24 ± 0.02

* Analysed from 27 micrographs

** Significant at $P < 0.05$ level

*** Significant at $P < 0.01$ level

Table 5:2

Effects of acclimation to hypoxic conditions on the fine structure of tench fast muscle fibres.

Parameter Mean ± S.E.	Environmental oxygen level $Po_2 \approx 21.0$KPa	 $Po_2 \approx 1.7$KPa
No. fibres analysed	66	58
Fibre area (μm^2)	666.26 ± 53	570.32 ± 43.7
Fibre perimeter (μm)	68.15 ± 2.8	96.47 ± 4.2
Fibre area occupied by subsarcolemmal zone (%)	14.8 ± 1.3	12.4 ± 1.0
No. subsarcolemmal mitochondria per fibre cross-section	15.8 ± 1.6	7.3 ± 0.9***
No. of interfibrillar mitochondria per fibre cross-section	2.3 ± 0.3	0.3 ± 0.1***
Fractional volume of mitochondria (total) (%)	4.5 ± 0.5	1.8 ± 0.3**
Fractional volume of subsarcolemmal mitochondria (%)	3.9 ± 0.5	1.8 ± 0.3**
Fractional volume of inter-fibrillar mitochondria (%)	0.6 ± 0.1	0.04 ± 0.02***
Fractional volume of myofibrils (%)	73.1 ± 0.8	82.7 ± 1.1**
Fractional volume of sarcoplasmic reticulum (%)*	3.2 ± 0.2	6.5 ± 0.3**
Fractional volume of T-system*	0.4 ± 0.05	0.26 ± 0.01

* Analysed from 27 micrographs

** Significant at $P < 0.05$ level

*** Significant at $P < 0.01$ level

Figure 5:1

The ultrastructure of the different muscle fibres in the tench myotome. A series of transverse sections of muscle from fish acclimated to aerated water.

(a) Superficial, small-diameter fibre from the slow fibre region (see Fig. 2:4). These fibres are characterised by a low mitochondrial content compared to slow fibres (b, c). Note the extensive subsarcolemmal space.

(b) Slow fibre from the lateral line triangle.

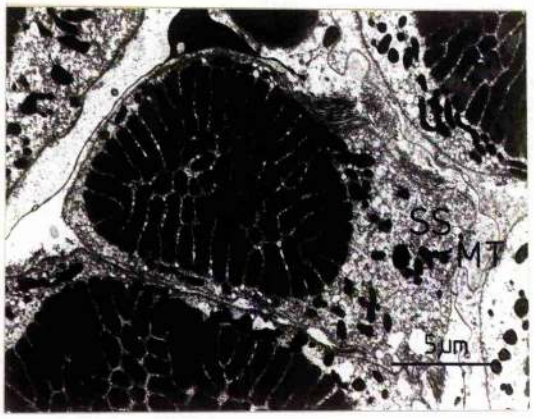
(c) Slow fibre from the lateral line triangle.

(d) An aerobic fast fibre. The mitochondrial volume fraction is somewhat lower than in the slow fibres (eg b, c) and the myofibrils are more regularly packed.

(e) Small-diameter fast glycolytic fibre showing invagination of the sarcolemma forming an insertion with the myosepta. Note the isolated bundles of myofibrils, extensive glycogen deposits and the presence of mitochondria with almost complete absence of internal structure.

(f) Fast glycolytic fibre. The subsarcolemmal zone is small and the mitochondria are sparse and small. Note the dense packing of myofibrils and the elongate peripheral myofibrils.

KEY: MY, myofibrils; MT, mitochondria; SS, subsarcolemmal space; C, capillaries; N, nucleus; G, glycogen granules; SF, sarcolemma.



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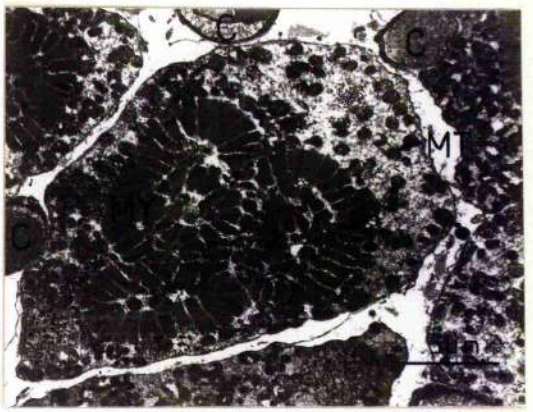

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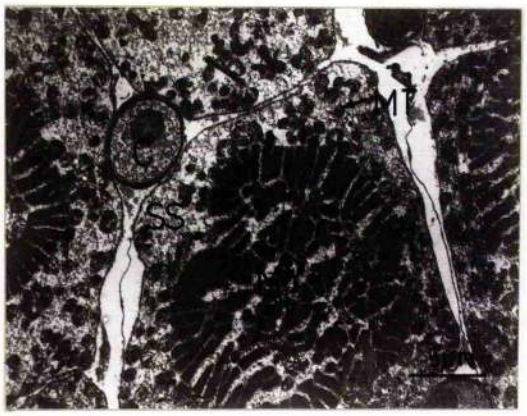

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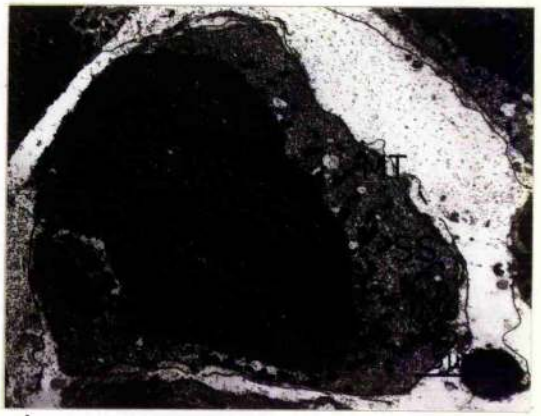

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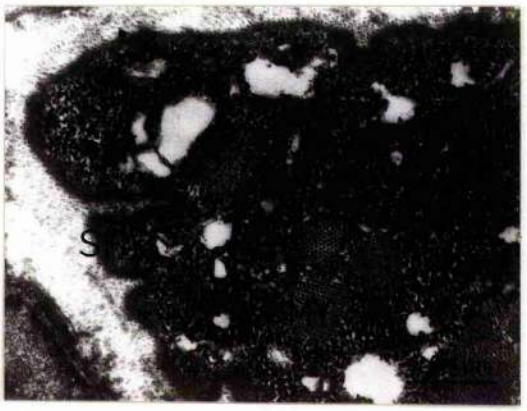

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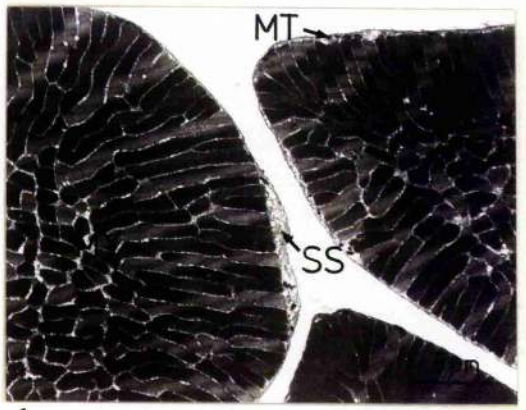

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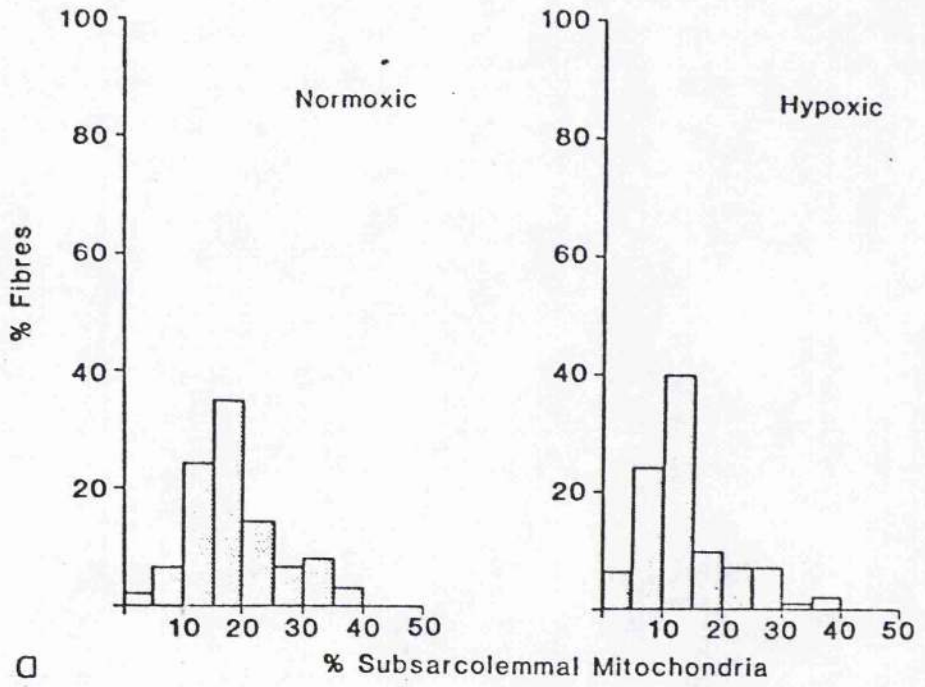
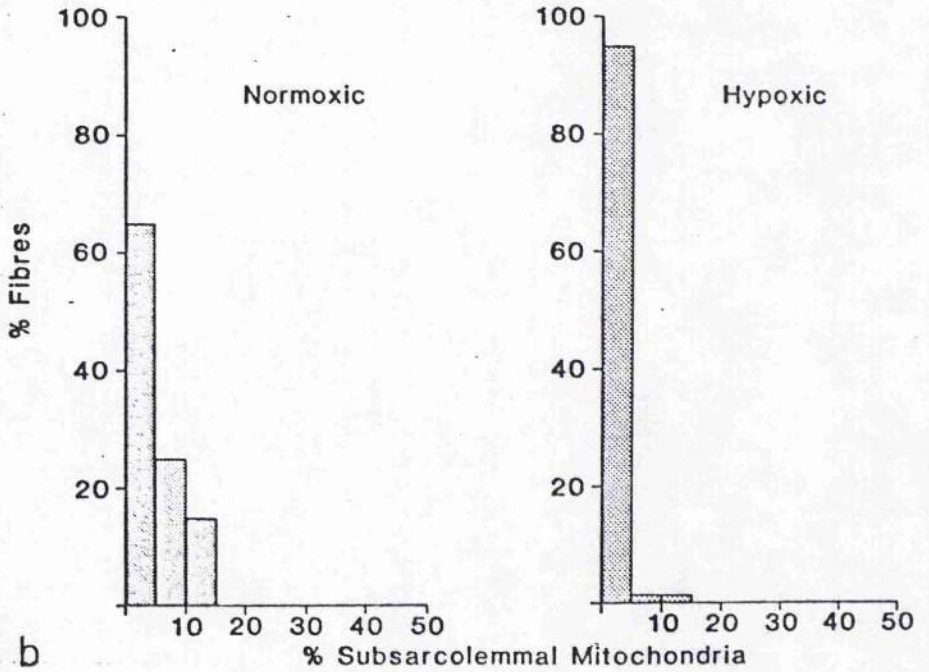
Figure 5:2

(a) Histograms showing the frequency distribution of subsarcolemmal mitochondria volume fractions (%) from slow muscle fibres isolated from tench acclimated to either aerated water (normoxic) or hypoxia.

(b) Histograms showing the frequency distribution of subsarcolemmal mitochondria volume fractions (%) for fast glycolytic fibres from tench acclimated to either aerated water (normoxic) or hypoxia.


Normoxic
Hypoxic
% Fibres
100
80
60
40
20
10 20 30 40 50
% Subsarcolemmal Mitochondria

a

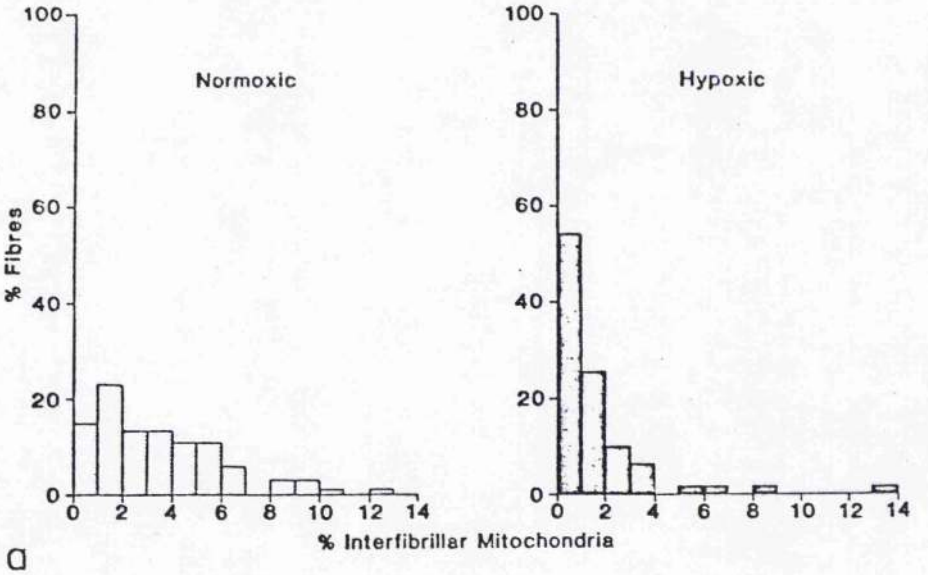

Normoxic
Hypoxic
% Fibres
100
80
60
40
20
10 20 30 40 50
% Subsarcolemmal Mitochondria

b

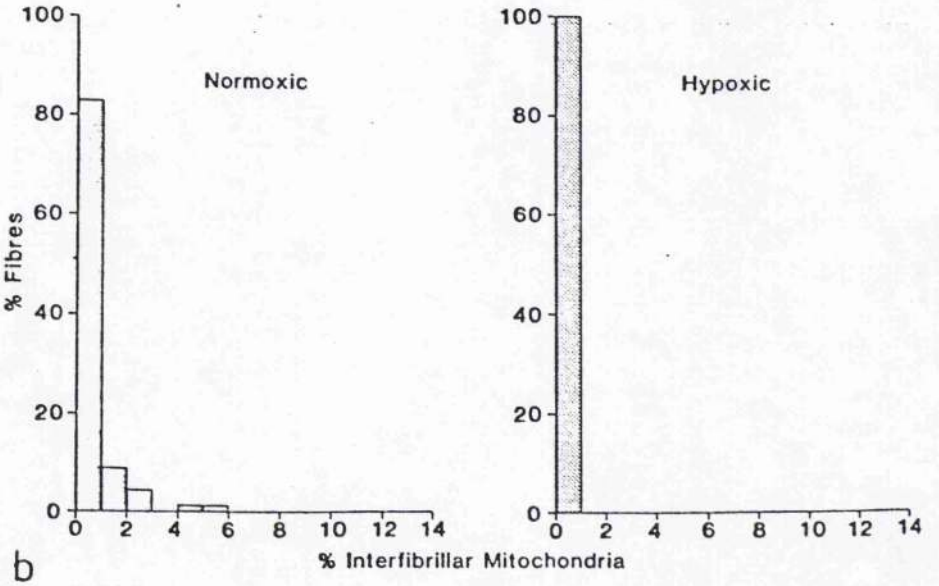
Figure 5:3

(a) Frequency distribution of interfibrillar mitochondrial volume fractions (%) for slow fibres from tench acclimated to either aerated water (normoxic) or hypoxia.

(b) Frequency distribution of interfibrillar mitochondrial volume fractions (%) for fast glycolytic fibres from tench acclimated to either aerated water (normoxic) or hypoxia.


Normoxic
Hypoxic
% Fibres
% Interfibrillar Mitochondria
0
20
40
60
80
100
0 2 4 6 8 10 12 14

a

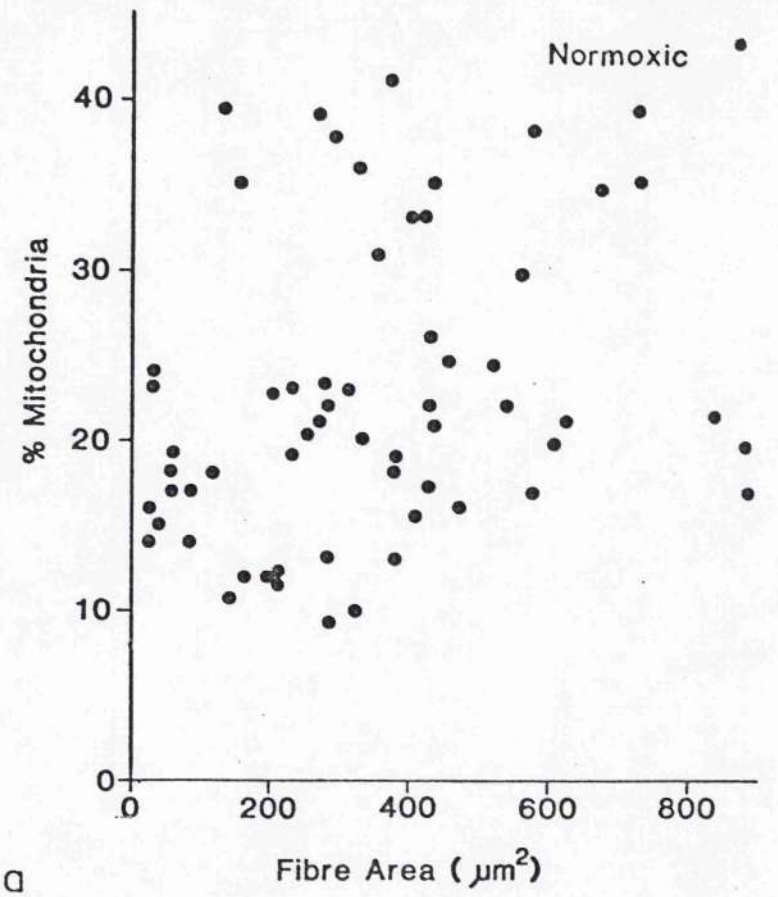

Normoxic
Hypoxic
% Fibres
% Interfibrillar Mitochondria
0
20
40
60
80
100
0 2 4 6 8 10 12 14

b

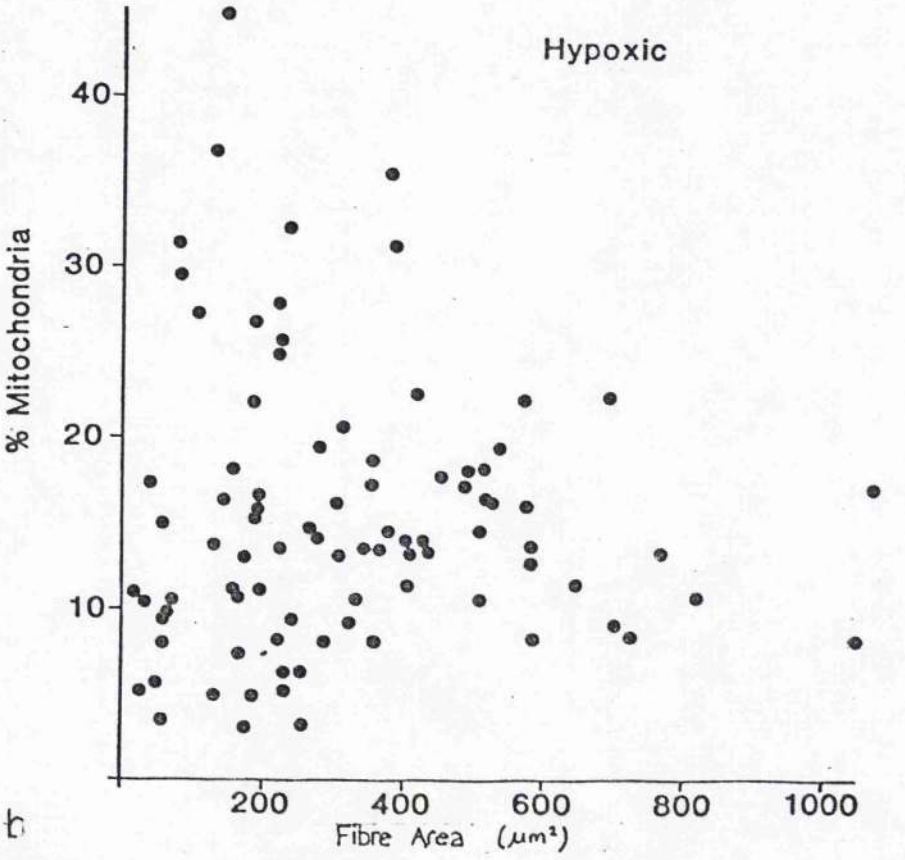
Figure 5:4A

(a) The relationship between fibre cross-sectional area (μm^2) and volume fractions of mitochondria (%) for slow muscles from tench acclimated to aerated water. Note the lack of correlation between fibre size and mitochondrial volume.

(b) The relationship between the fibre cross-sectional area (μm^2) and volume fractions of mitochondria for slow muscles from tench acclimated to hypoxic water. Note that the decrease in mitochondrial volume fraction following acclimation to low oxygen is not associated with any particular fibre size.


Normoxic
% Mitochondria
40
30
20
10
0
0
200
400
600
800
Fibre Area (μm²)

a

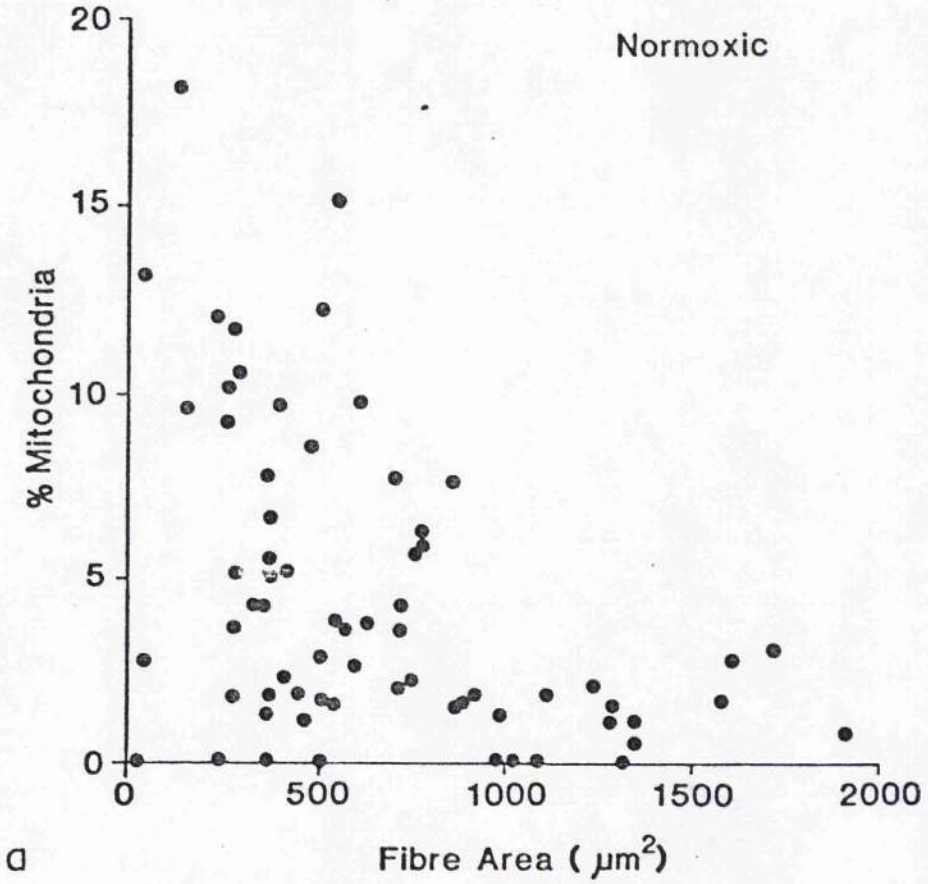

Hypoxic
% Mitochondria
40
30
20
10
200
400
600
800
1000
Fibre Area (μm²)

b

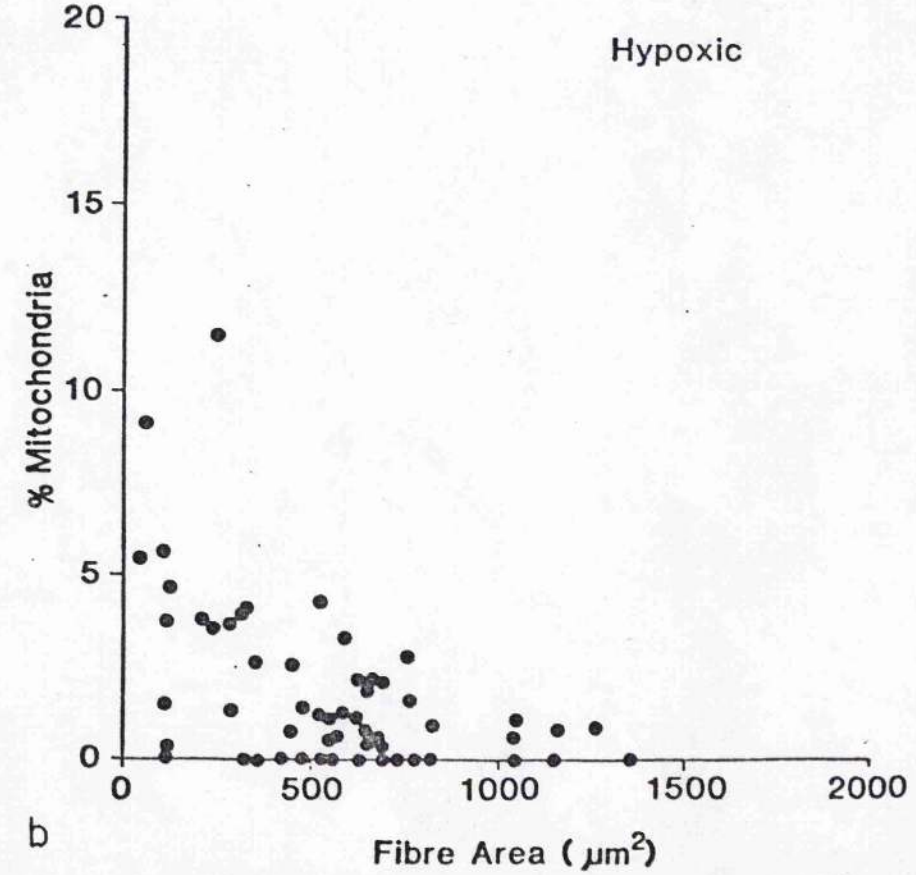
Figure 5:4B

(a) The relationship between fibre cross-sectional area (μm^2) and volume fractions of mitochondria (%) for fast glycolytic muscle from tench acclimated to aerated water. Note the tendency for fibres with areas less than 750 um^2 to have higher volume fractions of mitochondria than larger fibres.

(b) The relationship between fibre cross-sectional area (μm^2) and volume fractions of mitochondria from tench acclimated to hypoxic water. Note the decrease in mitochondrial volume compared with the fibres in (a).


Normoxic
% Mitochondria
20
15
10
5
0
0
500
1000
1500
2000
Fibre Area (μm²)

a

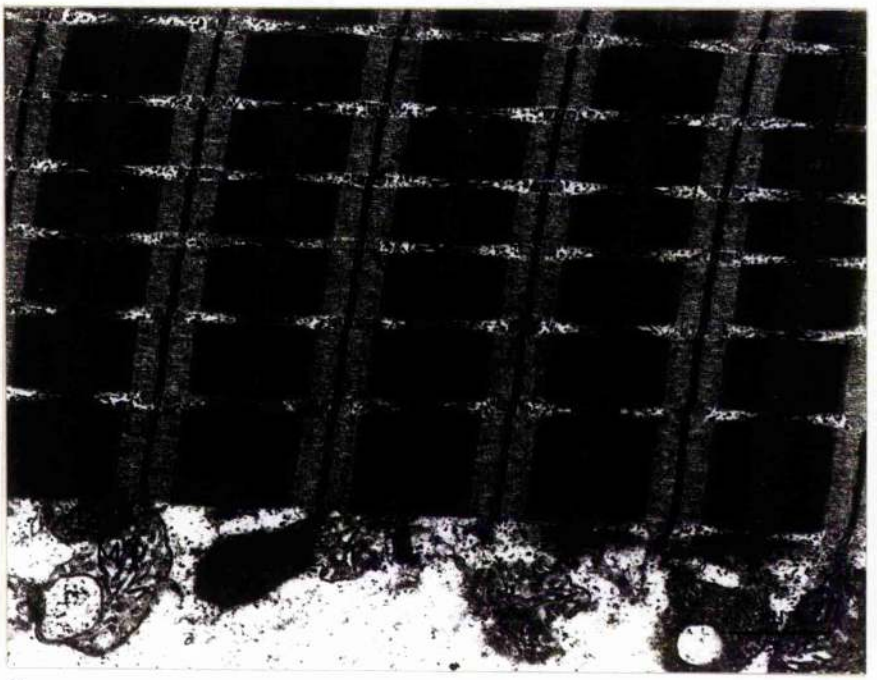

Hypoxic
% Mitochondria
20
15
10
5
0
0
500
1000
1500
2000
Fibre Area (μm²)

b

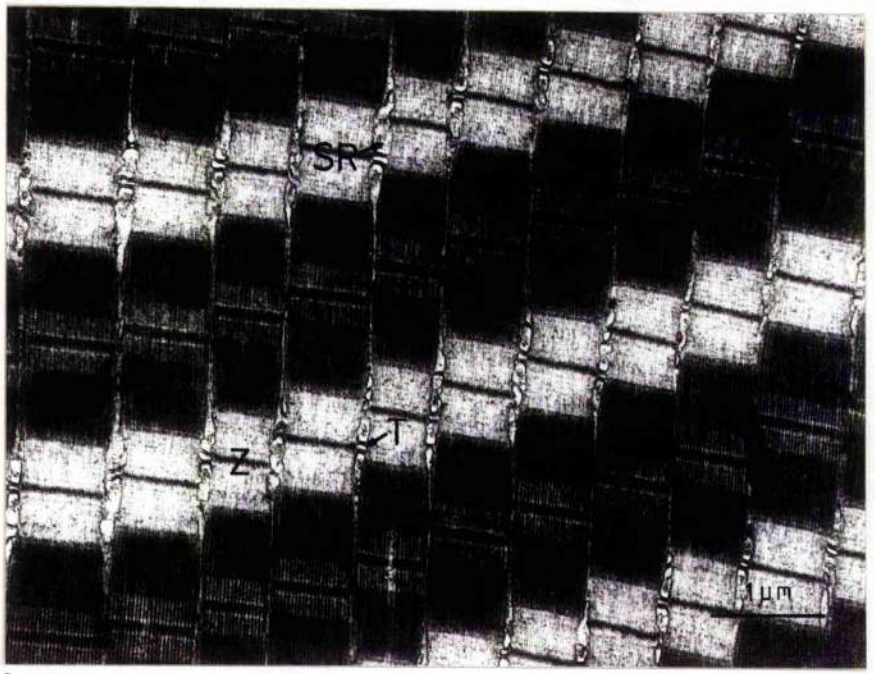
Figure 5:5

(a) Longitudinal section through a slow fibre from a tench acclimated to aerated water. Note the presence of a distinctive M-line (M) and triads (T) located at the level of the Z-disc (Z). MY, myofibrils; MT, mitochondria; G, glycogen granules; SR, sarcoplasmic reticulum.

(b) Longitudinal section through a fast fibre from a tench acclimated to aerated water. Note the larger amounts of sarcoplasmic reticulum (SR) compared to the slow fibre in (a). T, T-system; Z, Z-disc; MY, myofibrils; M, M-line.



a



b

Figure 5:6

The ultrastructure of the fast and slow fibres from the myotomal muscle of tench acclimated to hypoxic water.

(a) Small slow fibre.

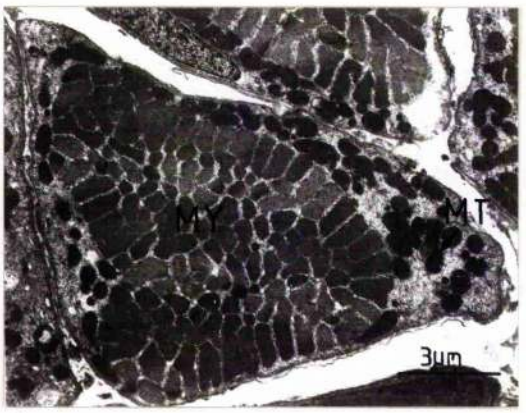
(b) Slow fibre. Note the variation in slow fibre structure (eg compared with (a) particularly with respect to the size of the subsarcolemmal zone. The fraction of fibre volume occupied by mitochondria is somewhat reduced compared to that of slow fibres from fish acclimated to aerated water (eg Fig. 5:1, a-c).

(c) Longitudinal section through the end of a slow fibre showing an extensive subsarcolemmal space, degenerating mitochondria and neuromuscular endplates.

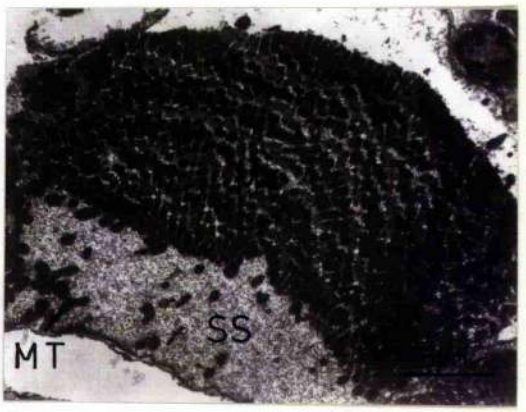
(d) Longitudinal section through a fast glycolytic fibre.

(e) Longitudinal section through a contracted slow fibre.

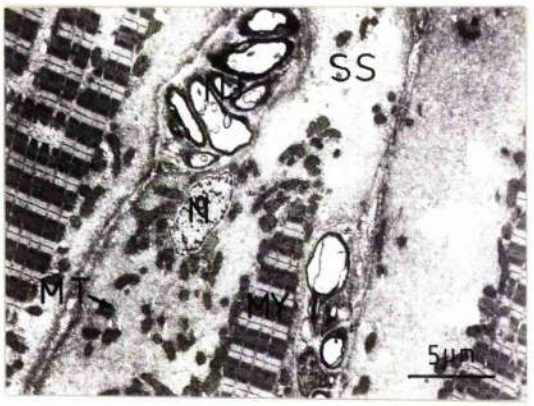
KEY: MY, myofibrils; MT, mitochondria; SS, subsarcolemmal space; N, nucleus; NJ, neuromuscular junction; Z, Z-disc; M, M-line; T, T-tubules; G, glycogen granules; SR, sarcoplasmic reticulum.



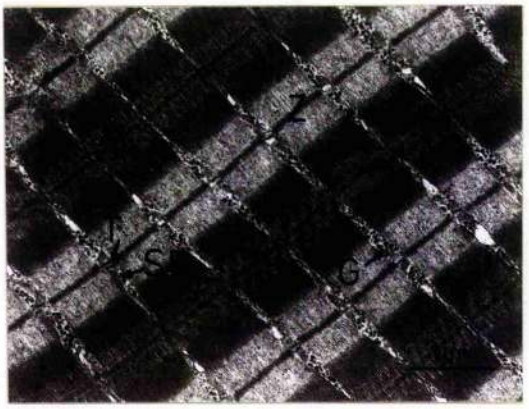
a



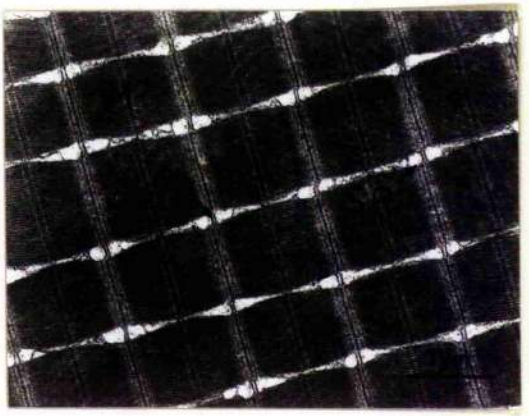
b



c



d



e

Figure 5:7

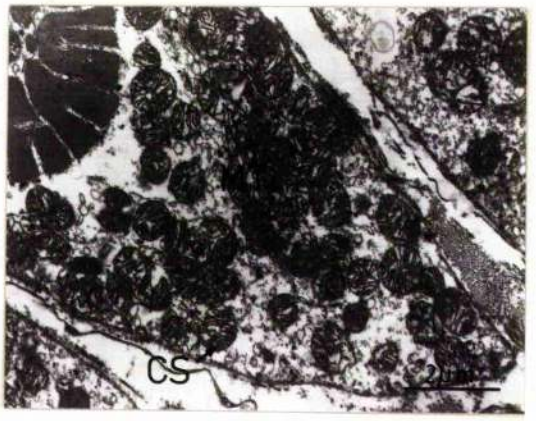
(a) High-magnification detail of the subsarcolemmal zone of a slow fibre from a tench acclimated to aerated water to show the typical structure of cristae (CS) of mitochondria (MT).

(b) Mitochondria (MT) from a slow fibre of a tench acclimated to aerated water. Note the densely packed cristae (CS). MY, myofibrils.

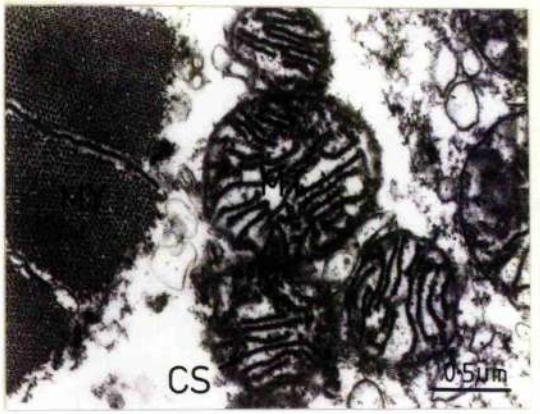
(c) Degenerating mitochondria (MT) from a slow muscle fibre from a tench acclimated to hypoxia showing the loss of cristae (CS).

(d) Transverse section through the peripheral myofibrils (MY) of a fast glycolytic fibre from a tench acclimated to hypoxia. Note the relatively poorly developed mitochondrial cristae (CS). This is also seen in a proportion of fast glycolytic fibres from fish acclimated to aerated water. MT, mitochondria.

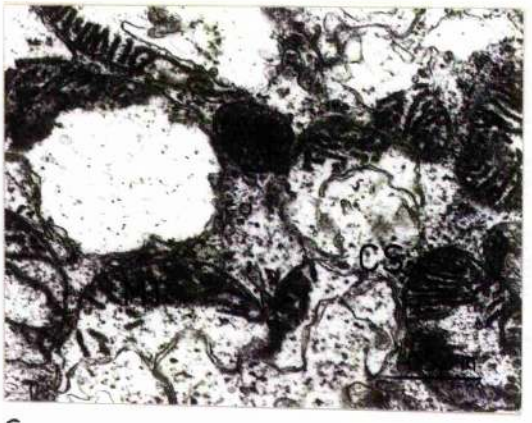
(e) Longitudinal section through the end region of a slow fibre from a tench acclimated to hypoxic water. The presence of an extensive network of endoplasmic reticulum indicates that this is a region of active longitudinal growth. Some fibrils that appear to be at an early stage of assembly are indicated by arrows. ER, endoplasmic reticulum; N, nucleus; MT, mitochondria; MY, myofibrils.



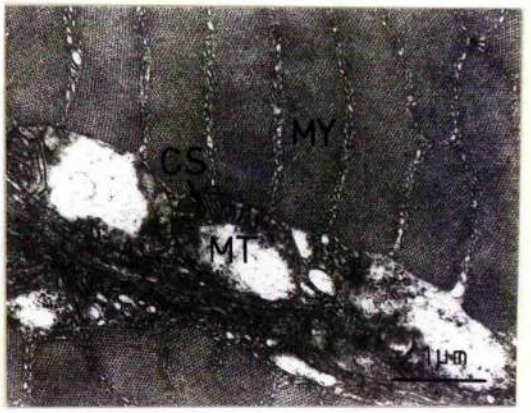
a



b



c



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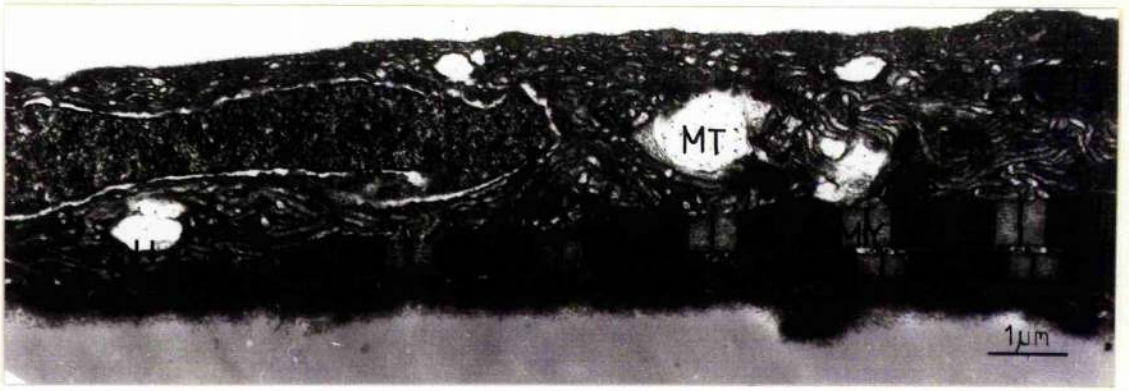
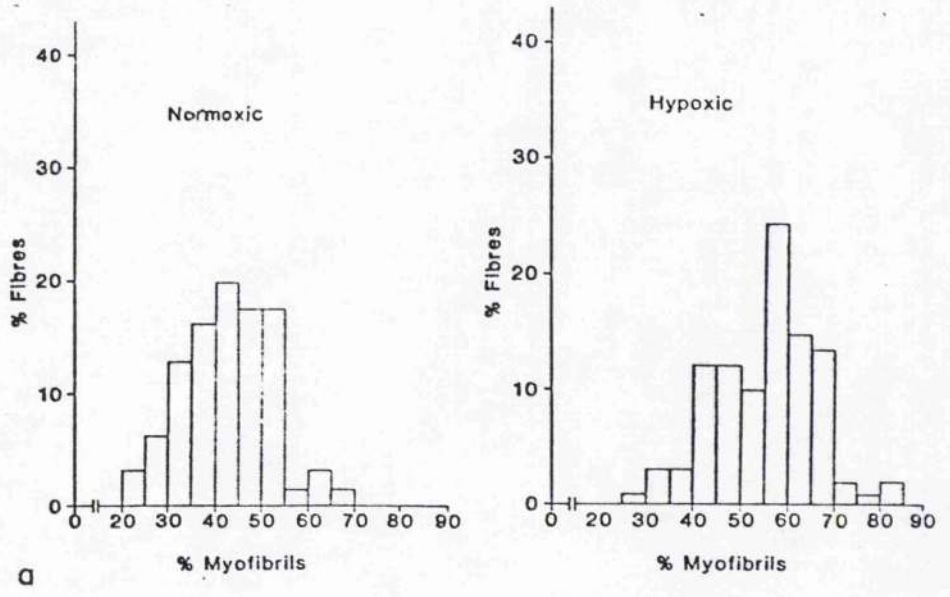


e

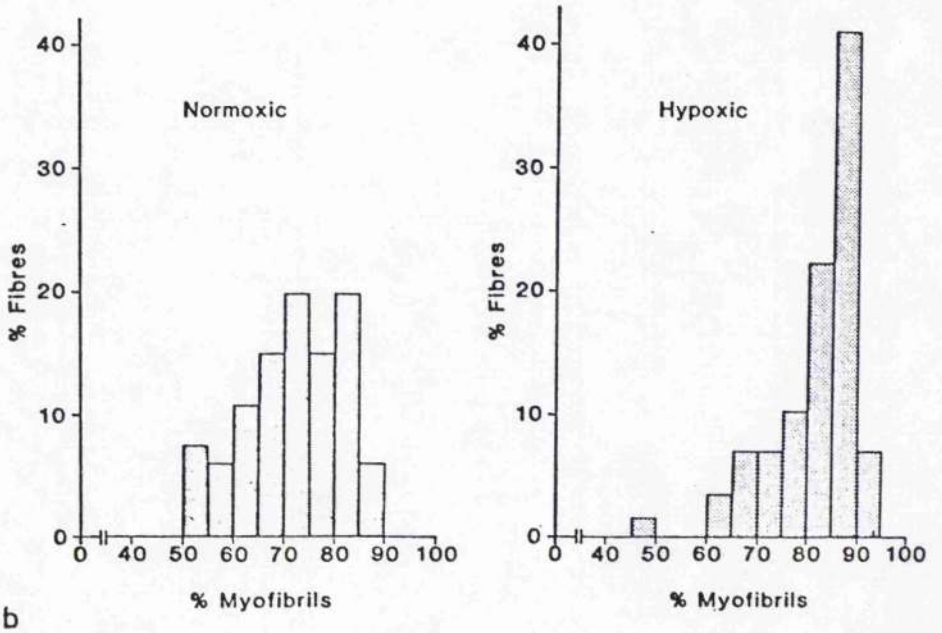
Figure 5:8

(a) Histograms showing the frequency distribution of volume fractions of myofibrils (%) for slow fibres of tench acclimated to aerated (normoxic) and hypoxic water.

(b) Histograms showing the frequency distribution of volume fractions of myofibrils (%) fast glycolytic fibres of tench acclimated to aerated (normoxic) and hypoxic water.


Normoxic
Hypoxic
% Fibres
% Myofibrils
0
10
20
30
40
0 20 30 40 50 60 70 80 90

a


Normoxic
Hypoxic
% Fibres
% Myofibrils
0
10
20
30
40
0 40 50 60 70 80 90 100

b

CHAPTER 6

CAPILLARY SUPPLY AND MITOCHONDRIAL VOLUME : THE AFFECTS OF ACCLIMATION TO HYPOXIA

Introduction

There are several factors which combine to determine the maximum aerobic capacity of a muscle. These include the volume density of mitochondria and the number of respiritory chains on the cristae of these mitochondria, capillary density, blood flow rate, blood perfusion distribution, myoglobin concentration and various factors influencing the haemoglobin-oxygen equilibrium such as the concentrations of the nucleoside triphosphates.

The morphometric parameters, volume density of mitochondria of fibres ($V_{v\ (m,\ f)}$) and capillary density ($N_{A\ (c,\ f)}$) set structural limits on oxygen demand and supply. Although they alone are insufficient parameters to assess the full functional significance of the capillary network they do provide some useful information on the oxygen demand and supply to skeletal muscles.

The original theory that there should be a critical capillary density below which some parts of a tissue may become anoxic was postulated by Krough in 1922. He considered muscle as a series of cylinders each supplied by a central capillary. Capillary supply is of particular importance in skeletal muscle since during exercise mitochondrial energy production can only be maintained if there is a sufficient continuous oxygen supply.

Mathieu et al., (1981) have shown that for a wide range of African mammals there is a close correlation between the volume density of

mitochondria in skeletal muscles and maximal oxygen uptake however this information cannot be assessed for fish due to the difficulties in obtaining reliable maximal respiration rates and thus the lack of data on the subject. Mitochondrial volume density is more loosely correlated with capillary density in a group of African mammals (Hoppeler et al., 1981). These parameters can be reliably determined in fish muscle and thus provide a useful correlation readily comparable between species.

It has been established that acclimation to many environmental factors can produce changes in the mitochondrial volume density and the capillary supply to skeletal muscle.

Exposure to low temperatures causes an increase in capillary density in the rat (Heroux & St. Pierre, 1957) and increases in capillary densities have been found in guinea pigs, rats and dogs exposed to simulated high altitude (Valdivia, 1958; Miller & Hale, 1970; Banchero et al., 1976; Eby & Banchero, 1976). In the fishes, modifications in both mitochondrial volume density and capillary density have been observed to occur in response to such factors as reduced food availability (Johnston, 1981b; Beardall & Johnston, 1982), temperature (Johnston, 1982a) and hypoxia (see Chapter 5). For instance acclimation of Crucian carp to temperatures of 28°C (summer temperature) produces a fall in $V_{v\ (m,\ f)}$ and $N_{A\ (c,\ f)}$ to 14.7% and 3121 respectively compared to 31.4% and 6383 for fish acclimated to 2°C (winter temperature) (Johnston, 1982a).

There have been a number of studies on the capillary supply to fast and slow muscles in fish (Kryvi et al., 1980; Totland et al., 1981; Egginton & Johnston, 1982b; Johnston, 1982a, c; Salamonski & Johnston, 1982), however few authors have made comparative studies of the effects of acclimation to variable environmental factors (Johnston, 1981b, 1982a; Beardall & Johnston, 1982) and no one has yet studied the

effects of acclimation to hypoxia on capillary supply and mitochondrial volume densities.

It appears that capillary supply to the fast muscle in fishes is related to the type of innervation. Capillary supply to multiply innervated fast fibres is significantly greater than for those that are terminally innervated by single neuromuscular endplates (Bone, 1978a; Johnston, 1981a).

Mitochondrial volume densities of fish slow muscle fibres have been found to be 3 - 10 times higher than for homologous muscles in birds and mammals (Eisenberg et al., 1974; Hoppeler et al., 1981a, b). This may reflect the fact that, despite composing only a small proportion of body weight, the slow muscle receives a relatively high proportion of cardiac output (Daxboeck et al., 1982). There is morphometric evidence to suggest that the volume density of mitochondria, as measured on serial sections of identical muscle fibres, is constant (James & Meek, 1976; Pette et al., 1980), thus it seems reasonable to assume that aerobic capacity of a single fibre remains constant along its length and that estimates of mitochondrial volume densities taken from transverse sections give a true representation.

Capillary densities ($N_{A\ (c,\ f)}$) and mitochondrial volume densities ($V_{v\ (m,\ f)}$) of the red and white muscles have been determined for all three species acclimated to both aerated and hypoxic water. Capillary densities were expressed as the number of capillary profiles counted ($N_{(c)}$) per unit cross-sectional area of muscle fibres ($A_{(f)}$) as this has the advantage of avoiding inherent preservation errors of the extracellular space and relates the capillaries to the same reference space as the mitochondria.

There are many difficulties involved in directly comparing the results of one author to those of another. Individuals vary in their

experimental techniques and methods of assessing capillary supply and often sample numbers are too small to give a reliable result. Methological difficulties in assessing capillary supply to fish muscle have been discussed by Egginton and Johnston (1983).

Materials and Methods

The tench, Tinca tinca, L.

Sixteen fish of mean length ($\pm$ S.E.) 16.0 $\pm$ 0.7 cm were divided into two groups and acclimated to either aerated or hypoxic water for six weeks as described in Chapter 2. Methods of quantification are detailed in Chapter 2.

The actual numbers of fibres analysed for capillary supply and mitochondrial volume density were as follows; from fish acclimated to aerated water 115 slow, 67 fast and from fish acclimated to hypoxic water 67 slow and 99 fast (Tables 6:1, 6:2). Different numbers in each subgroup arose as a consequence of the random sampling technique employed. The final mean values for the parameters measured by planimetry varied by < 5% from the mean values of the first 25 randomly selected fibres in each subgroup. This would indicate that the minimum number of sections analysed to obtain a representative sample has been exceeded by a reasonable safety margin.

The Crucian carp, Carassius carassius L.

Sixteen Crucian carp of mean body weight ($\pm$ S.E.) 73.5 $\pm$ 2.8 g were divided into two groups and acclimated to aerated and hypoxic water for six weeks as described in Chapter 2.

Twenty five fibres from each subgroup were analysed for mitochondrial volume density and capillary supply was determined from 159 slow and 90 fast fibres from aerated water acclimated fish and from 185 slow and

122 fast fibres from hypoxic water acclimated animals (Tables 6:3, 6:4).

The catfish, Clarias mossambicus, Richter

Sixteen catfish of mean body weight ($\pm$ S.E.) 50.0 $\pm$ 5.3 g were divided into two groups and acclimated to aerated or hypoxic water for 27 days as described in Chapter 2.

The mitochondrial volume density was determined from twenty five fibres from each subgroup and capillary supply from 162 slow and 122 fast fibres from fish acclimated to aerated water and 168 and 118 fast fibres from fish acclimated to hypoxic water (Tables 6:5, 6:6).

Preparation for ultrastructural examination and morphological and quantitative techniques for all three species are described in Chapter 2.

Results

The tench, Tinca tinca, L.

Mitochondrial volume densities of the red and white muscle fibres from tench acclimated to aerated or hypoxic water have already been discussed in Chapter 5. Acclimation to hypoxia results in a decrease in V_v (m, f) from 0.23 to 0.15 in slow fibres (Table 6:1) and from 0.045 to 0.018 in fast fibres (Table 6:2). Mitochondria are mainly concentrated in the subsarcolemmal zone (Table 6:7). The frequency distribution of the percentage of fibre volume equal to mitochondria is shown for slow muscle of both acclimation groups in Figure 6:1 and for fast muscle in Figure 6:2.

Mean fibre cross-sectional area was unchanged in both slow and fast muscle after acclimation to hypoxia (Tables 6:1, 6:2), as was the frequency distribution of fibre size (Fig. 6:3, 6:4).

In both groups total mitochondrial volume density was around 8 times higher in slow than in fast muscle and 74% of the aerated acclimated fishes slow fibre mitochondria were found in the subsarcolemmal zone. This fell to 64% in the hypoxia acclimated animals indicating a slight rise in the fractional volume of intermyofibrillar mitochondria (Table 6:7). Figure 6:1 shows a displacement to the right, in the hypoxic acclimated carp, of the frequency distribution of the percentage of fibre volume occupied by mitochondria.

The mean fibre cross-sectional area was not changed much for either fibre type by acclimation to hypoxia (Tables 6:3, 6:4) and their frequency distributions were reasonably similar (Figs. 6:3, 6:4) and cover wide ranges, especially in the fast fibres.

The measured and calculated indices of capillarisation for Crucian carp acclimated to aerated and hypoxic water are given in Tables 6:3 and 6:4. Around 67% of fast muscle and 25% of slow muscle fibres have no direct capillary contact in the aerated water acclimated group. Acclimation to hypoxia did not alter this significantly. It fell to 17% in slow muscle and remained at 67% in fast (Table 6:3, 6:4). The average number of capillaries touching each fibre increased slightly for slow muscles (1.5 to 1.9) but was unchanged for fast muscles (0.35 to 0.36) following acclimation to hypoxia (Tables 6:3, 6:4; Figs. 6:5, 6:6). Frequency distributions were very similar for both acclimation groups in the case of the fast fibres (Fig. 6:6) however they were somewhat different for the slow fibres (Fig. 6:5).

The volume density of capillaries was around 7 times higher in slow than fast muscles in both acclimation groups. There was a small though insignificant increase in $N_{A\ (c,\ f)}$ after acclimation to hypoxia from 1639 to 1811 in slow muscle and from 240 to 260 in fast muscle (Tables 6:3, 6:4).

The effects of hypoxia acclimation on $V_{v\ (m,\ f)}$ and $N_{A\ (c,\ f)}$ are shown diagramatically in Figure 6:7 for slow muscle and Figure 6:8 for fast muscle. Some of the ultrastructural features of carp muscle are illustrated in Figure 6:10.

The catfish, Clarias mossambicus, Richter.

Total mitochondrial volume densities in catfish acclimated to aerated water were 0.16 for and 0.025 for fast muscle fibres (Tables 6:5, 6:6). Following acclimation to hypoxia there was little change in these numbers, the slow fibres rising slightly, though insignificantly to 0.19 and the fast remaining the same (Tables 6:5. 6:6). Almost all the mitochondria were situated in the subsarcolemmal zone in both fibre types (Table 6:7). The frequency distributions of the percentage of fibres occupied by mitochondria are shown in Figures 6:1 and 6:2.

Figures 6:3 and 6:4 show the distribution of fibre size in fast and slow muscle fibre populations. Both fibre types have a wide range of fibre cross-sectional areas and acclimation to hypoxia results in a 36% decrease in the mean cross-sectional area of fast muscle fibres ($P < 0.05$) (Table 6:6).

The various measured and calculated indices of capilliarisation are shown in Tables 6:5 and 6:6. In fish acclimated to aerated water there were around nine times as many capillaries in slow muscle compared to fast. The mean number of capillaries touching each slow fibre dropped slightly from 1.9 in aerated water acclimated fish to 1.4 in hypoxic acclimated animals (Table 6:5). The frequency distribution of the number of capillaries touching each muscle fibre for fish acclimated to aerated and hypoxic water are shown in Figures 6:5 and 6:6.

Essentially hypoxia acclimation caused no significant changes in any of the various indices of capillary supply investigated. $N_{A\ (c,\ f)}$ was 1633 in the slow muscle of aerated water acclimated fish and 1657

The percentage of fibres without direct capillary contact in fish acclimated to aerated water was found to be 14% for slow fibres and 38% for fast fibres (Tables 6:1, 6:2). Acclimation to hypoxia resulted in a large increase in these numbers to 31% in slow fibres and 82% in fast (Tables 6:1, 6:2). The average number of capillaries touching each fibre was reduced from 1.7 to 0.9 in slow and from 0.8 to 0.2 in fast fibres (Tables 6:1, 6:2) and the frequency distribution of the number of capillaries touching each fibre was correspondingly displaced to the left in both cases (Fig. 6:5, 6:6). The effects of acclimation to hypoxia on various other measured and calculated indices of capillary supply to tench slow and fast glycolytic muscle are given in Tables 6:1 and 6:2. Capillary density ($N_{A\ (c,\ f)}$) is markedly decreased by hypoxia acclimation, falling from 2672 to 1371 in slow muscle and from 676 to 250 in fast, decreases of 49% and 63% respectively. The effects of hypoxia acclimation on mitochondrial density ($V_{v\ (m,\ f)}$) and capillary density ($N_{A\ (c,\ f)}$) for slow and fast muscles are shown diagramatically in Figures 6:7 and 6:8.

Capillary cross-sectional area decreases slightly in both fibre types after acclimation to hypoxia. Capillary cross-sectional areas are generally very consistent in size, varying little from the mean. This is illustrated in the frequency distribution of capillary cross-sectional area in Figure 6:9.

The Crucian carp, Carassius carassius, L.

Following acclimation to hypoxia there was a significant increase in mitochondrial volume density in both fast and slow muscle fibres in the Crucian carp. In slow muscle $V_{v\ (m,\ f)}$ rosefrom 0.15 in aerated water to 0.25 in hypoxic water acclimated animals (Table 6:3). A smaller increase from 0.018 to 0.03 was observed in the fast muscle of

in hypoxic water acclimated animals. In fast muscle $N_{A\ (c,\ f)}$ was 189 and 136 respectively.

The results of hypoxia acclimation on $N_{A\ (c,\ f)}$ and $V_{v\ (m,\ f)}$ are summarised diagramatically in Figures 6:7 and 6:8.

Discussion

The results demonstrate that the effects of hypoxia acclimation on fish muscle vary considerably between species. The way in which mitochondrial volume densities and muscle capillary supply is affected by hypoxia acclimation varies between the three species depending upon the behavioral, respiratory and cardiovascular adjustments made to reduced P_{O_2} as well as other unmeasured parameters. Slow fibres from all three species studied acclimated to aerated water have higher mitochondrial volume densities than fast fibres (Tables 6:1 - 6:6) and also substantially greater capillary supply (Tables 6:1 - 6:6) indicating a more highly developed capacity for aerobic metabolism. There is a reasonable correlation between the mitochondrial content of fish slow muscle and sustained swimming performance. For example active and continuous swimming species such as the anchovy, Engraulis encrasicolus and the Atlantic mackeral, Scomber scomber, have slow muscle mitochondrial volume densities of 46% and 36% respectively (Bone, 1978a; Johnston, 1982b), whereas the Rat fish, Chimaera monstrosa, which mainly uses its fins for swimming has a slow muscle mitochondrial volume density of only 5% (Totland et al., 1981). The tench, Crucian carp and catfish are all relatively sedentary species and do not swim continuously to any extent. Their slow muscle mitochondrial volume densities are relatively midway between those of active and sluggish species (23%, 15% and 16% respectively) (Tables 6:1, 6:3, 6:5), reflecting their habits. There is also a tendancy for cold adapted

species to have higher mitochondrial volume densities (Johnston, 1982a; Walesby & Johnston, 1980).

Little is known about the mechanisms responsible for changes in capillary density and size of mitochondrial compartment so explanations are somewhat speculative and can only be discussed in relation to the parameters studied ie oxygen consumption, behavioral responses and fibre types. There is still much scope for further work on the effects of acclimation to hypoxia on such factors as the concentrations of nucleoside triphosphates in the red cells and biochemistry before a complete picture of what is happening can be built up.

In the tench hypoxia caused $V_{v\ (m,\ f)}$ to fall by 35% and 60% in slow and fast fibres respectively. This was paralleled by a 49% fall in $N_{A\ (c,\ f)}$ in the slow fibres and 63% in the fast and a fall in oxygen consumption to 48% of that of aerated acclimated animals. It would appear that these responses would be more detrimental than beneficial however it does seem likely that, in the wild, they would occur during the animals hibernation period, when it is exposed to hypoxia buried in the mud. Here it would not be moving around, merely 'ticking over' so the muscles would not require a large oxygen supply. The capillary supply would still be able to supply sufficient oxygen for basal needs and the remaining mitochondria sufficient energy. Routine oxygen requirements would be much reduced during hibernation. The enhanced capacity for glycolysis and for fatty acid oxidation (Johnston & Bernard, 1982b) would serve to supply any sudden short term energy needs by reducing the threshold for anaerobic metabolism and also would allow some tolerance should the hypoxia become more severe.

A similar observation has been made by Hudlicka et al., (1973). He found that in kittens, during muscle development, as glycolytic activity increased blood flow decreased however the reverse did not

occur ie glycolytic enzyme activity did not decrease with increasing blood flow. It may be, therefore, that in tench the enhanced capacity for glycolysis (Johnston & Bernard, 1982b) causes a decrease in blood flow and thus atrophy of capillaries.

The response of the Crucian carp is very much the opposite to that of the tench. Although capillary supply stayed much the same in both fibre types mitochondrial volume density was increased by 40% in both the red and white fibres. The same sort of rise in $V_{v\ (m,\ f)}$ is seen in cold acclimated Crucian carp (Johnston, 1982a) however here it is accompanied by a large increase in capillary density. There is a significant increase in oxygen consumption in Crucian carp following acclimation to hypoxia (see Chapter 3). Routine oxygen consumption of Crucian carp in aerated water and of hypoxic acclimated fish in hypoxic water was around twice as much as tench at the same temperature (see Chapter 3). Crucian carp do not hibernate during exposure to hypoxia but maintain activity, though this is generally reduced (Lomholt & Johansen, 1978). It is possible that the large increase in mitochondrial volume may represent an adaptation by which the animal increases the uptake of oxygen from the muscle capillaries thus decreasing the oxygen level of the venous blood returning to the gills. This would increase the rate of oxygen loading at the gills due to the greater difference between water and gill P_{O_2}'s. Crucian carp do not hibernate so it would be necessary to maintain an adequate supply of oxygen to meet routine needs during hypoxia.

Van den Thillart has measured changes in enzyme activity after acclimation to hypoxia at two different temperatures in a species closely related to the Crucian carp, the goldfish, Carassius auratus. At higher temperatures hypoxia acclimation leads to increased oxidative capacity in muscle tissues while the anaerobic capacity remains unchanged.

This would tie in with the increased mitochondrial volume seen in the Crucian carp after hypoxic acclimation.

Similar effects have been found in a number of mammals subject to endurance training exercise (Saulbert, 1973; Holloszy & Booth, 1976). Training resulted in a significant increase in oxidative capacity and no change in glycolytic capacity in fast twitch muscles in the rat (Saulbert, 1973; Holloszy & Booth, 1976). Gollnick and King (1979) reported an increase in the number and size of mitochondria in rats after training. In rabbits, chronic electrical stimulation of twitch muscles produces significant increases in capillary density after 4 days followed by increases in mitochondrial capacity and aerobic enzyme activity (Brown et al., 1976). These changes in mammalian muscle may reflect either an altered pattern of motor neurone firing or else an increase in contractile activity per se. It is possible that increased perfusion results in elevated tissue P_{O_2} which in turn stimulates mitochondrial biogenesis (Brown et al., 1976; Hudlicka et al., 1977).

There is no significant change in either mitochondrial volume density or capillary density in catfish red and white fibres after acclimation to hypoxia. The obvious explanation for this lies in the fact that the catfish has an alternative means of obtaining oxygen by air breathing so it does not have to rely entirely upon its aquatic environment as its oxygen source. It would seem that maximal oxygen demands can be met by a combination of increased frequency and volume of ventilation of the supra-branchial organs and adjustments in the efficiency of oxygen extraction at the gills (see Chapter 3). It has been shown for Clarias mossambicus that total respiration rate is 22% higher for fish acclimated to hypoxia than for fish subject to acute hypoxia (see Chapter 3). This is largely a result of a 164% increase in aquatic respiration. If maximal routine oxygen demands can be met

by simply altering the respiration rate the need for structural changes is obliviated.

One structural parameter which was significantly changed in hypoxia acclimated Clarias mossambicus was the mean cross-sectional area of the fast fibres (Table 6:6). This decreased from a mean of 1265μm^2 in aerated water to 809 μm^2 in hypoxic water (Table 6:6). It has been demonstrated that forced swimming and exercise over a period of time causes muscle fibre hypertrophy in fish (Greer-Walker, 1971; Johnston & Moon, 1980a, b) therefore it seems likely that a reduction in locomotory activity will cause fibre atrophy as for example happens in the muscles of limbs immobilised by being encased in plaster after bone breakage. It was observed that during hypoxia spontaneous locomotory activity was reduced. This might have caused a decrease in mean fibre cross-sectional area due to fibre atrophy as well as reducing routine oxygen requirements.

There is a good correlation between capillary density, $N_{A\ (c,\ f)}$ and volume density of mitochondria, $V_{v\ (m,\ f)}$, for the slow muscle fibres of the three species studied and of several other species studied by the same methods (Fig. 6:11, Johnston, 1982a; Egginton & Johnston, 1982a, b; Salamonski & Johnston, 1982). These two parameters are almost linearly related and it would appear that the surface area of the capillary network available for gas exchange is a limiting factor in determining the size of the mitochondrial compartment and hence aerobic capacity. A similar correlation has been found in the limb muscles of some African mammals (Hoppelar et al., 1981) though this relationship is more scattered due to the muscles containing a mixture of fibre types.

Table 6:1

Effects of acclimation to hypoxic conditions on the capillary supply to slow muscle fibres in the Tench, Tinca tinca.

Parameter Mean ± S.E.	Symbol/ Calculation	Acclimation condition aerated water $P_{O_2} \approx 17.6$ KPa	hypoxic water $P_{O_2} \approx 1.5$ KPa
No. of fibres analysed	A	115	67
Percentage of fibres without direct capillary contact	B	14	31
No.of capillaries associated with fibres analysed	C	106	34
Fibres cross-sectional area (μm^2)	D	345 ± 24	370 ± 24
Fibre circumference (μm)	E	70 ± 3	73.7 ± 2.6
Volume density of mitochondria	F	0.23 ± 0.01	0.15 ± 0.01
Average number of capillaries per fibre	G	1.73 ± 0.11	0.94 ± 0.10
Fibre circumference in direct contact with capillaries (μm)	H_1	12.9 ± 1.0	8.84 ± 0.8
	H_2	11.1 ± 1.0	6.07 ± 0.7
Percentage of fibre circumference in direct contact with capillaries	I_1	17.2 ± 1.3	11.10 ± 0.9
	I_2	14.6 ± 1.2	7.6 ± 0.9
Capillary circumference (μm) per μm^2 of fibre area (H/D)	J_1	0.037	0.024
	J_2	0.032	0.016
Area of capillary wall supplying 1 μm^3 of mitochondria (J/F)	K_1	0.16	0.16
	K_2	0.14	0.11
Cross-sectional area of capillaries (μm^2)	$\bar{a}$ (c)	21.76 ± 2.4	15.67 ± 1.5
Circumference of capillaries (μm)	$\bar{b}$ (c)	19.40 ± 1.5	16.39 ± 0.8
Number of capillaries per unit volume of muscle fibres (mm^{-2})	NA (c, f)	2672	1371

1. Mean values calculated for vascularised fibres only.

2. Mean values for all fibres

Table 6:2

Effects of acclimation to hypoxic conditions on the capillary supply to the fast muscle fibres in the Tench, *Tinca tinca*.

Parameter Mean ± S.E.	Symbol/ Calculation	Acclimation condition aerated water	hypoxic water
No. of fibres analysed	A	67	99
Percentage of fibres without direct capillary contact	B	38	82
No. of capillaries associated with fibres analysed	C	50	21
Fibres cross-sectional area (μm^2)	D	838 ± 117	846 ± 62
Fibre circumference (μm)	E	115 ± 7	113 ± 4
Volume density of mitochondria	F	0.045 ± 0.005	.018 ± 0.003
Average number of capillaries per fibre	G	0.8 ± 0.9	0.2 ± 0.05
Fibre circumference in direct contact with capillaries (μm)	H_1	6.4 ± 0.6	5.9 ± 1.4
	H_2	4.1 ± 0.5	1.0 ± 0.3
Percentage of fibre circumference in direct contact with capillaries	I_1	6.8 ± 0.8	5.5 ± 0.6
	I_2	4.2 ± 0.6	0.9 ± 0.2
Capillary circumference (μm) per um^2 of fibre area (H/D)	J_1	0.0076	0.0070
	J_2	0.0049	0.0012
Area of capillary wall supplying 1 μm^3 of mitochondria (J/F)	K_1	0.17	0.39
	K_2	0.11	0.07
Cross-sectional area of capillaries (μm^2)	$\bar{a}$ (c)	16.3 ± 2.5	12.67 ± 5.2
Circumference of capillaries (μm)	$\bar{b}$ (c)	17.47 ± 1.4	15.1 ± 2.9
Number of capillaries per unit volume of muscle fibres (mm^{-2})	NA (c, f)	676	250

1. Mean values calculated for vascularised fibres only.

2. Mean values for all fibres.

Table 6:3

Effects of acclimation to hypoxic conditions on the capillary supply to slow muscle fibres of the Crucian carp, Carassius carassius.

Parameter Mean ± S.E.	Symbol/ Calculation	Acclimation condition aerated water $P_{O_2} \approx 17.6$ KPa	hypoxic water $P_{O_2} \approx 1.5$ KPa
No. of fibres analysed	A	159	185
Percentage of fibres without direct capillary contact	B	25	17
No. of capillaries associated with fibres analysed	C	147	199
Fibres cross-sectional area (μm^2)	D	564 ± 38	594 ± 38
Fibre circumference (μm)	E	93 ± 3	102 ± 2
Volume density of mitochondria	F	0.15 ± 0.01	0.25 ± 0.01
Average number of capillaries per fibre	G	1.5 ± 0.09	1.9 ± 0.08
Fibre circumference in direct contact with capillaries (μm)	H_1	11.2 ± 0.7	16.0 ± 0.7
	H_2	9.4 ± 0.7	14.2 ± 0.7
Percentage of fibre circumference in direct contact with capillaries	I_1	11.5 ± 0.6	15.2 ± 0.6
	I_2	9.7 ± 0.6	13.5 ± 0.7
Capillary circumference (μm) per μm^2 of fibre area (H/D)	J_1	0.020	0.027
	J_2	0.017	0.024
Area of capillary wall supplying 1 μm^3 of mitochondria (J/F)	K_1	0.13	0.11
	K_2	0.11	0.10
Cross-sectional area of capillaries (μm^2)	$\bar{a}$ (c)	20.34 ± 1.4	18.30 ± 2.6
Circumference of capillaries (μm)	$\bar{b}$ (c)	18.66 ± 1.1	17.75 ± 0.75
Number of capillaries per unit volume of muscle fibres (mm^{-2})	NA (c, f)	1639	1811

1. Mean values calculated for vascularised fibres only.

2. Mean values for all fibres.

Table 6:4

Effects of acclimation to hypoxic conditions on the capillary supply to fast muscle fibres of the Crucian carp, Carassius carassius.

Parameter Mean ± S.E.	Symbol/ Calculation	Acclimation condition aerated water $P_{O_2} \approx 17.6$ KPa	hypoxic water $P_{O_2} \approx 1.5$ KPa
No. of fibres analysed	A	90	122
Percentage of fibres without direct capillary contact	B	67	67
No. of capillaries associated with fibres analysed	C	27	33
Fibres cross-sectional area (μm^2)	D	1267 ± 145	1040 ± 86
Fibre circumference (μm)	E	129 ± 6	117 ± 5
Volume density of mitochondria	F	0.018 ± 0.002	0.03 ± 0.003
Average number of capillaries per fibre	G	0.35 ± 0.05	0.36 ± 0.05
Fibre circumference in direct contact with capillaries (μm)	H_1	8.49 ± 0.9	7.38 ± 0.6
	H_2	2.83 ± 0.5	2.42 ± 0.4
Percentage of fibre circumference in direct contact with capillaries	I_1	7.26 ± 0.9	6.32 ± 0.6
	I_2	2.34 ± 0.5	2.27 ± 0.3
Capillary circumference (um) per μm^2 of fibre area (H/D)	J_1	0.007	0.007
	J_2	0.002	0.002
Area of capillary wall supplying 1 μm^3 of mitochondria (J/F)	K_1	0.39	0.23
	K_2	0.11	0.06
Cross-sectional area of capillaries (μm^2)	$\bar{a}$ (c)	22.65 ± 2.67	19.29 ± 1.8
Circumference of capillaries (μm)	$\bar{b}$ (c)	20.23 ± 1.23	18.97 ± 1.0
Number of capillaries per unit volume of muscle fibres (mm^{-2})	NA (c, f)	240	260

1. Mean values calculated for vascularised fibres only.

2. Mean values for all fibres.

Table 6:5

Capillary supply to slow myotomal muscle fibres of African catfish (*Clarias mossambicus*) acclimated to either aerated ($P_{O_2} \approx$ 15.1 KPa) or hypoxic water ($P_{O_2} \approx$ 2.4 KPa).

Parameter Mean ± S.E.	Symbol/ Calculation	Acclimation condition aerated water	hypoxic water
No. of fibres analysed	A	162	158
Percentage of fibres without direct capillary contact	B	13.6	8.9
No. of capillaries associated with fibres analysed	C	174	151
Fibres cross-sectional area (μm^2)	D	660 ± 30	581 ± 33
Fibre circumference (μm)	E	106 ± 3	96 ± 3
Volume density of mitochondria	F	0.16 ± 0.01	0.19 ± 0.01
Average number of capillaries touching each fibre	G	1.9 ± 0.1	1.4 ± 0.1
Fibre circumference in direct contact with capillaries (μm)	H_1	14.3 ± 0.8	11.6 ± 0.7
	H_2	12.4 ± 0.8	9.6 ± 0.7
Percentage of fibre circumference in direct contact with capillaries	I_1	13.3 ± 0.7	12.2 ± 0.7
	I_2	11.5 ± 0.7	10.2 ± 0.7
Capillary circumference (μm) per um^2 of fibre area (H/D)	J_1	0.021	0.019
	J_2	0.019	0.017
Area of capillary wall supplying 1 μm^3 of mitochondria (J/F)	K_1	0.13	0.10
	K_2	0.12	0.09
Cross-sectional area of capillaries (μm^2)	$\bar{a}$ (c)	20.3 ± 1.5	18.3 ± 1.1
Circumference of capillaries (μm)	$\bar{b}$ (c)	18.7 ± 0.7	17.7 ± 0.8
Number of capillaries per unit volume of muscle fibres (mm^{-2})	NA (c, f)	1633	1657

1. Mean values calculated for vascularised fibres only.

2. Mean values for all fibres.

Table 6:6

Capillary supply to fast myotomal muscle fibres of African catfish (*Clarias mossambicus*) acclimated to either aerated ($P_{O_2} \approx$ 15.1 KPa) or hypoxic water ($P_{O_2} \approx$ 2.4 KPa).

Parameter Mean ± S.E.	Symbol/ Calculation	Acclimation condition aerated water	hypoxic water
No. of fibres analysed	A	122	118
Percentage of fibres without direct capillary contact	B	72.2	80.5
No. of capillaries associated with fibres analysed	C	29	15
Fibre cross-sectional area (μm^2)	D	1265 ± 83	809 ± 63
Fibre circumference (μm)	E	149 ± 6	114 ± 4
Volume density of mitochondria	F	0.025 ± 0.002	0.024 ± 0.002
Average number of capillaries touching each fibre	G	0.3 ± 0.05	0.2 ± 0.04
Fibre circumference in direct contact with capillaries (μm)	H_1	6.99 ± 0.31	5.89 ± 0.22
	H_2	1.94 ± 0.33	1.14 ± 0.23
Percentage of fibre perimeter in direct contact with capillaries (H/E x 100)	I_1	5.0 ± 0.3	5.7 ± 0.2
	I_2	1.39 ± 0.25	1.11 ± 0.25
Capillary circumference per μm^2 of fibre area (H/D)	J_1	0.0055	0.0073
	J_2	0.0015	0.0014
Area of capillary wall supplying 1 μm^3 of mitochondria (J/F)	K_1	0.22	0.30
	K_2	0.061	0.059
Cross-sectional area of capillaries (μm^2)	$\bar{a}$ (c)	20.3 ± 2.5	19.7 ± 2.1
Circumference of capillaries (μm)	$\bar{b}$ (c)	18.7 ± 1.0	18.5 ± 0.9
Number of capillaries per unit volume of muscle fibres (mm^{-2})	NA (c, f)	189	136

1. Mean values calculated for vascularised fibres only.

2. Mean values for all fibres.

Table 6:7

Distribution of mitochondria in the slow and fast muscles of fish acclimated to either aerated or hypoxic water. Values represent mean ± S.E.

V_v (mt, f)	Slow muscle Acclimation condition		Fast muscle Acclimation condition	
	aerated water	hypoxic water	aerated water	hypoxic water
TENCH				
Subsarcolemmal zone	0.185 ± 0.009	0.138 ± 0.007	0.039 ± 0.005	0.018 ± 0.003
Intermyofibrillar zone	0.044 ± 0.006	0.012 ± 0.002	0.006 ± 0.001	0.0004 ± 0.0002
CRUCIAN CARP				
Subsarcolemmal zone	0.11 ± 0.008	0.16 ± 0.009	0.015 ± 0.002	0.022 ± 0.002
Intermyofibrillar zone	0.04 ± 0.005	0.09 ± 0.005	0.004 ± 0.001	0.009 ± 0.002
CATFISH				
Subsarcolemmal zone	0.12 ± 0.01	0.14 ± 0.02	0.023 ± 0.002	0.023 ± 0.001
Intermyofibrillar zone	0.03 ± 0.003	0.05 ± 0.05	0.002 ± 0.0007	0.0016 ± 0.0011

Figure 6:1

Frequency histograms of the distribution of the mitochondrial volume fractions (%) from the slow fibres of tench, Crucian carp and catfish acclimated to either aerated (Normoxic) or hypoxic water.

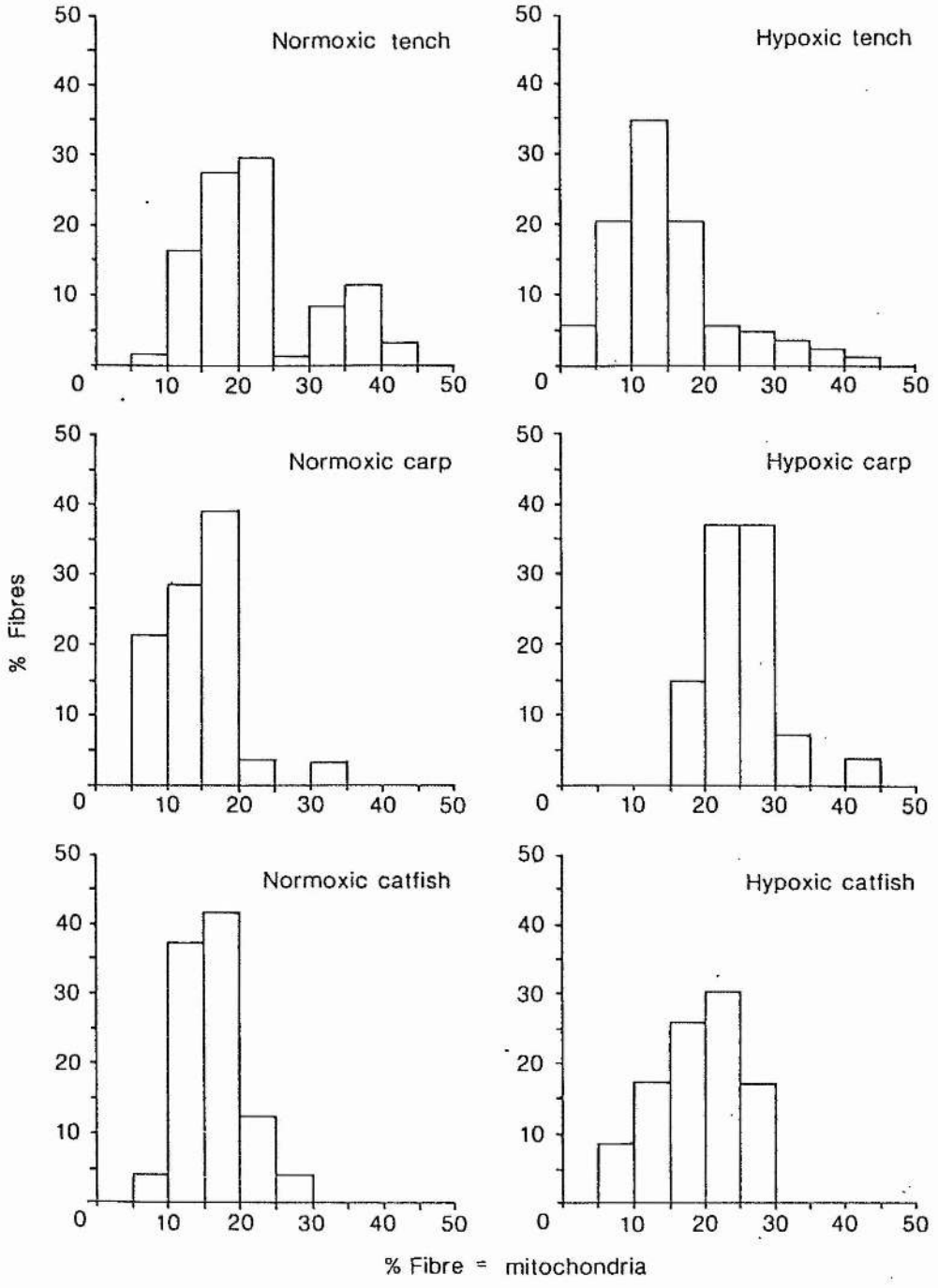

Normoxic tench
Hypoxic tench
Normoxic carp
Hypoxic carp
Normoxic catfish
Hypoxic catfish
% Fibres
% Fibre = mitochondria
0
10
20
30
40
50

Figure 6:2

Frequency histograms of the distribution of the mitochondrial volume fractions (%) from the fast glycolytic fibres of tench, Crucian carp and catfish acclimated to either aerated (Normoxic) or hypoxic water.

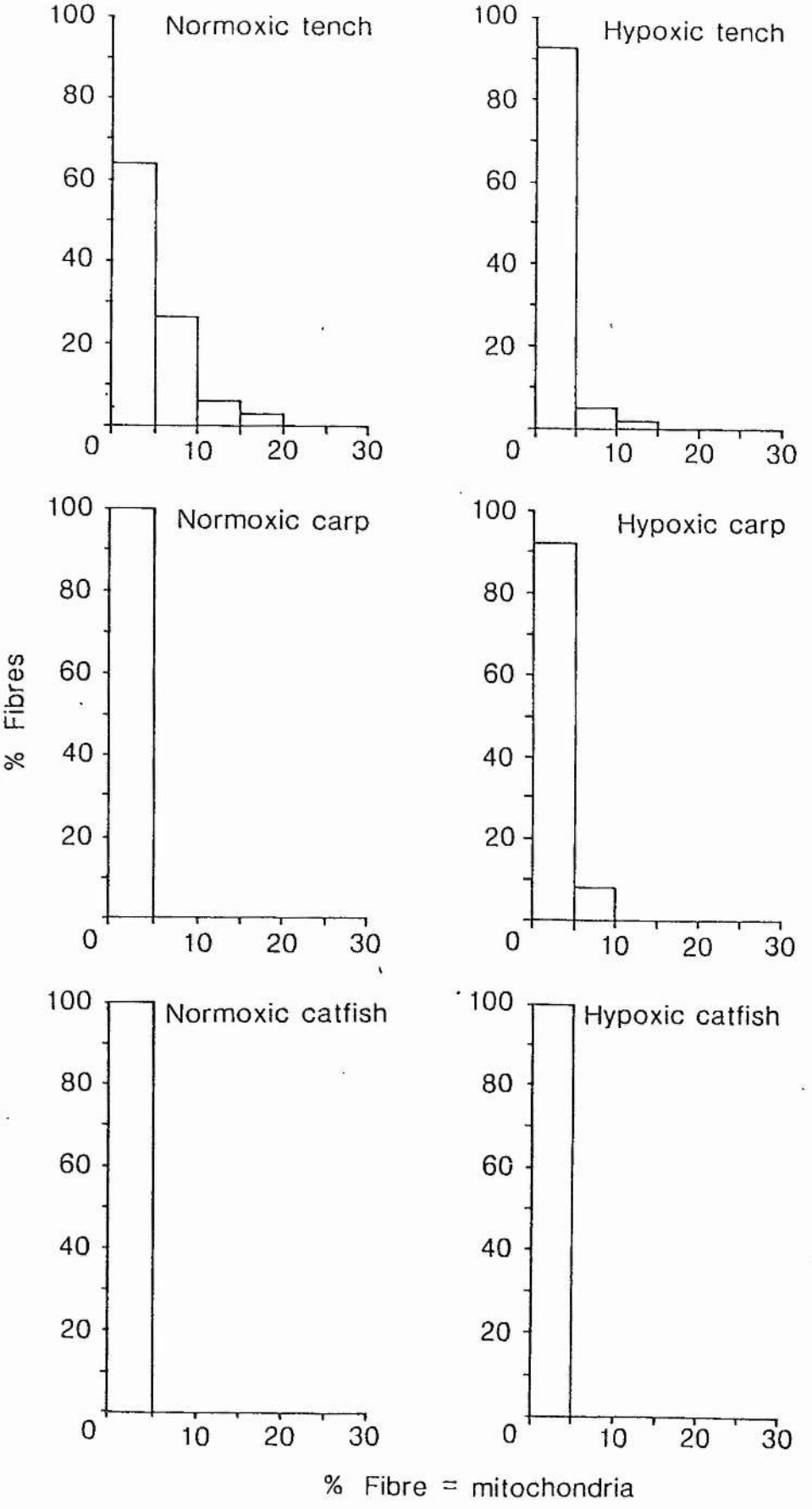

Normoxic tench
Hypoxic tench
Normoxic carp
Hypoxic carp
Normoxic catfish
Hypoxic catfish
% Fibres
100
80
60
40
20
0
10
20
30
% Fibre = mitochondria

Figure 6:3

Frequency histograms of the distribution of fibre size (cross-sectional area, μm^2) in slow muscles of tench, Crucian carp and catfish acclimated to aerated (Normoxic) and hypoxic water.

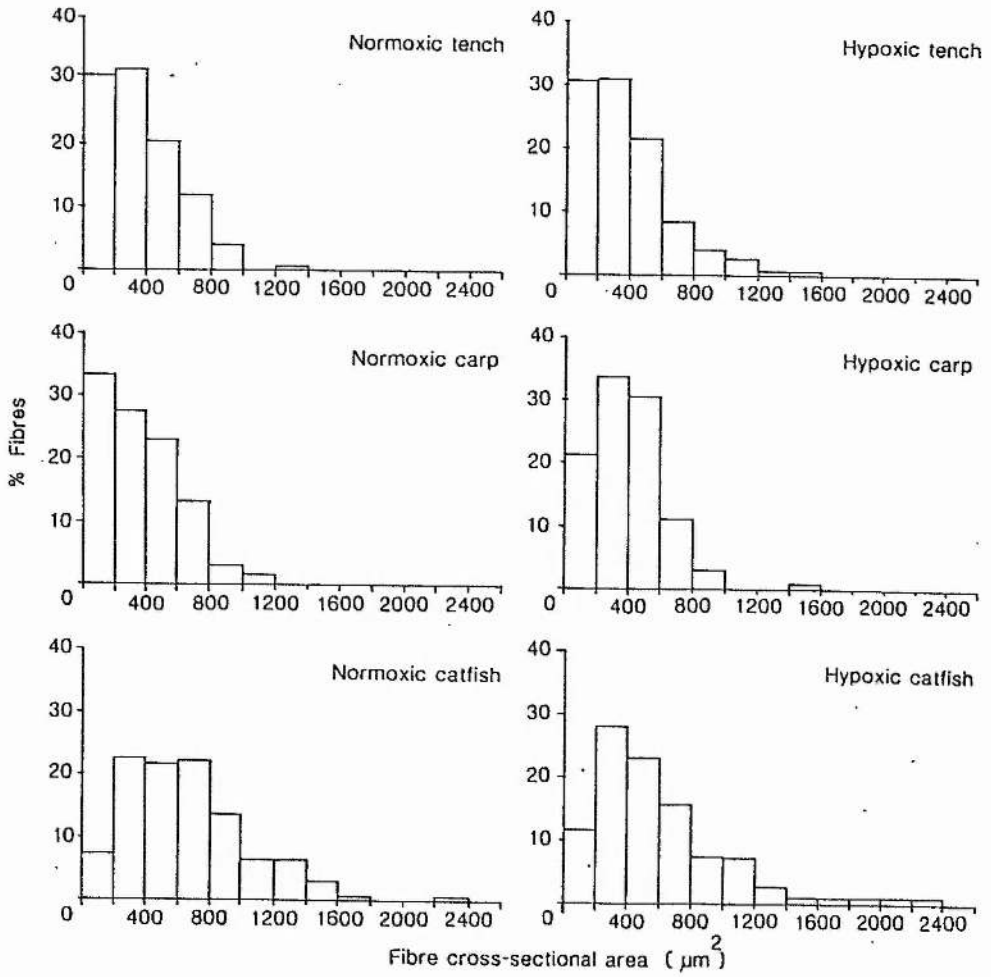

Normoxic tench
Hypoxic tench
Normoxic carp
Hypoxic carp
Normoxic catfish
Hypoxic catfish
% Fibres
0
10
20
30
40
400
800
1200
1600
2000
2400
Fibre cross-sectional area (µm 2)

Figure 6:4

Frequency histograms of distribution of fibre size (cross-sectional area, μm^2) in fast glycolytic muscles of tench, Crucian carp and catfish acclimated to aerated (Normoxic) and hypoxic water.

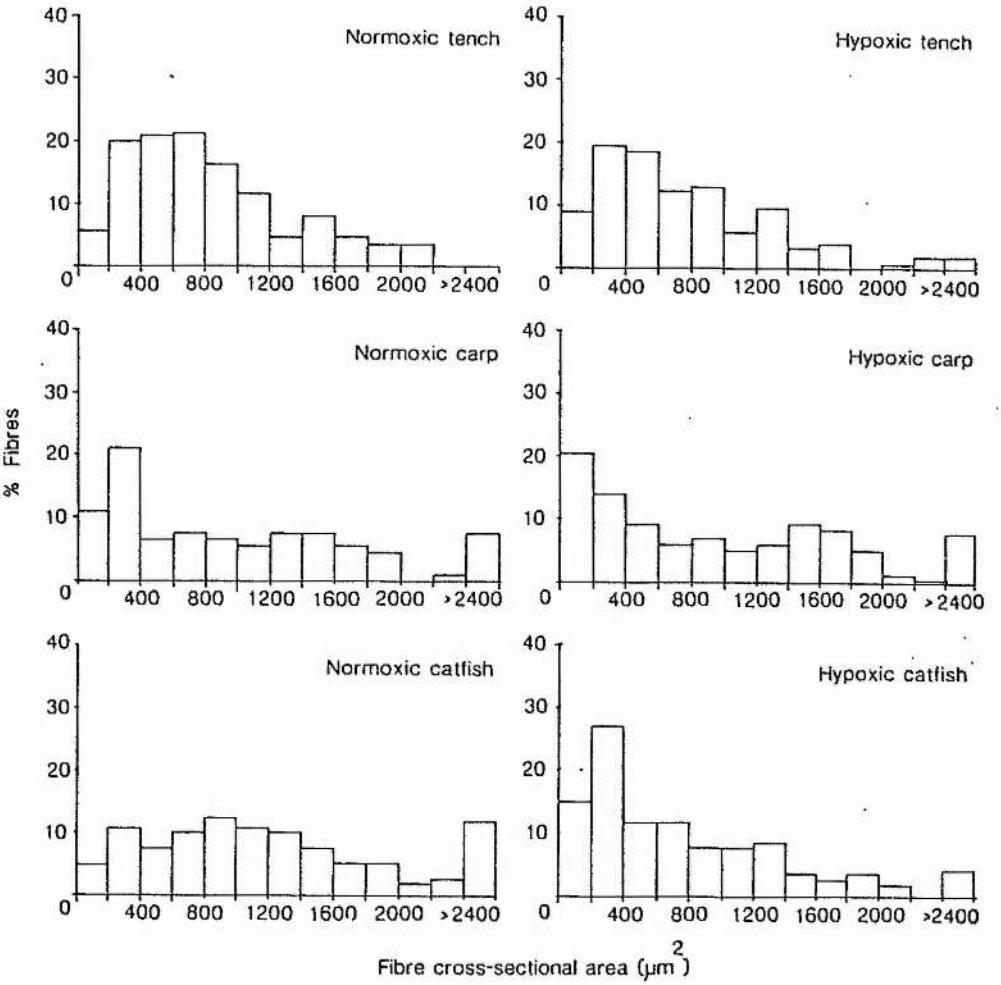

Normoxic tench
Hypoxic tench
Normoxic carp
Hypoxic carp
Normoxic catfish
Hypoxic catfish
% Fibres
0
10
20
30
40
400
800
1200
1600
2000
>2400
Fibre cross-sectional area (μm²)

Figure 6:5

Histograms showing the frequency distribution of the number of capillaries touching each fibre for slow myotomal muscle of the tench, Crucian carp and catfish acclimated to aerated (Normoxic) or hypoxic water (Hypoxic).

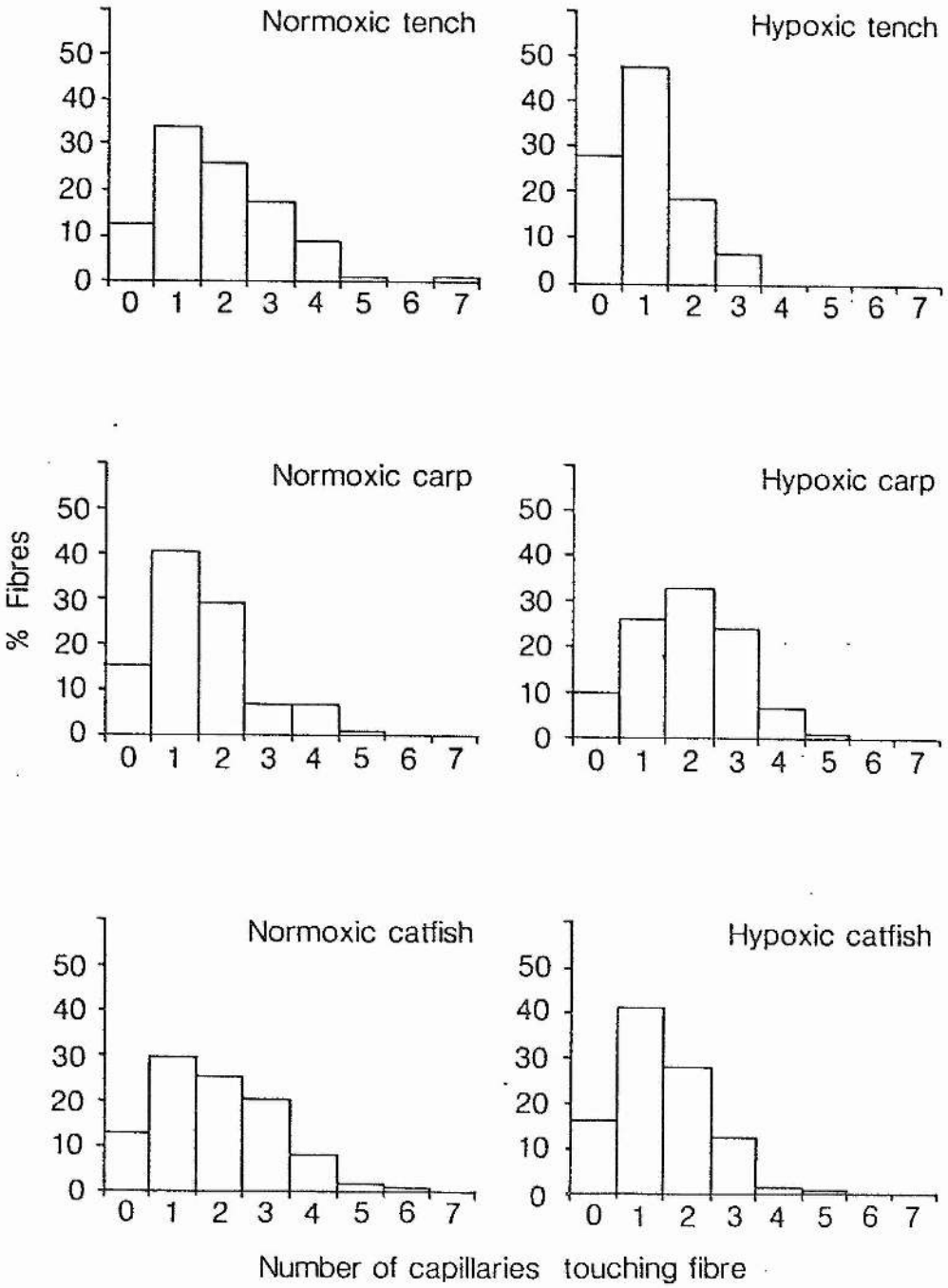

Normoxic tench
Hypoxic tench
Normoxic carp
Hypoxic carp
Normoxic catfish
Hypoxic catfish
% Fibres
0
10
20
30
40
50
0 1 2 3 4 5 6 7
Number of capillaries touching fibre

Figure 6:6

Histograms showing the frequency distribution of the number of capillaries touching each fibre for fast glycolytic muscle of the tench, Crucian carp and catfish acclimated to aerated (Normoxic) or hypoxic water (Hypoxic).

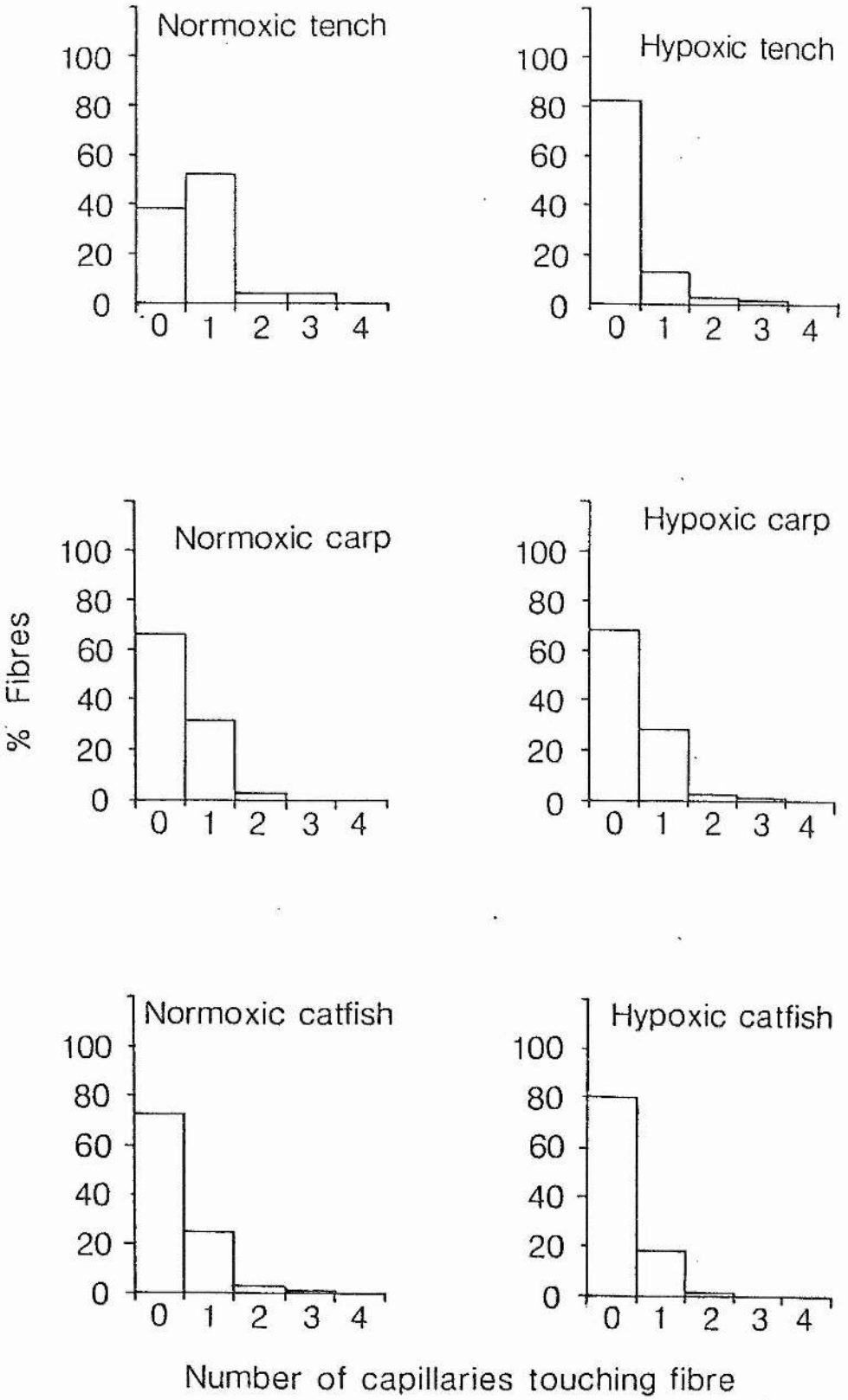

Normoxic tench
Hypoxic tench
Normoxic carp
Hypoxic carp
Normoxic catfish
Hypoxic catfish
% Fibres
100
80
60
40
20
0
0 1 2 3 4
Number of capillaries touching fibre

Figure 6:7

Histograms showing the effects of acclimation to hypoxic water on the mitochondrial volume density ($V_{v\ (mit)}$) and capillary density ($N_{a\ (c,\ f)}$) of the slow muscles of the tench, Crucian carp and catfish. The figures above each bar represent the P_{O_2} at which the fish were acclimated.

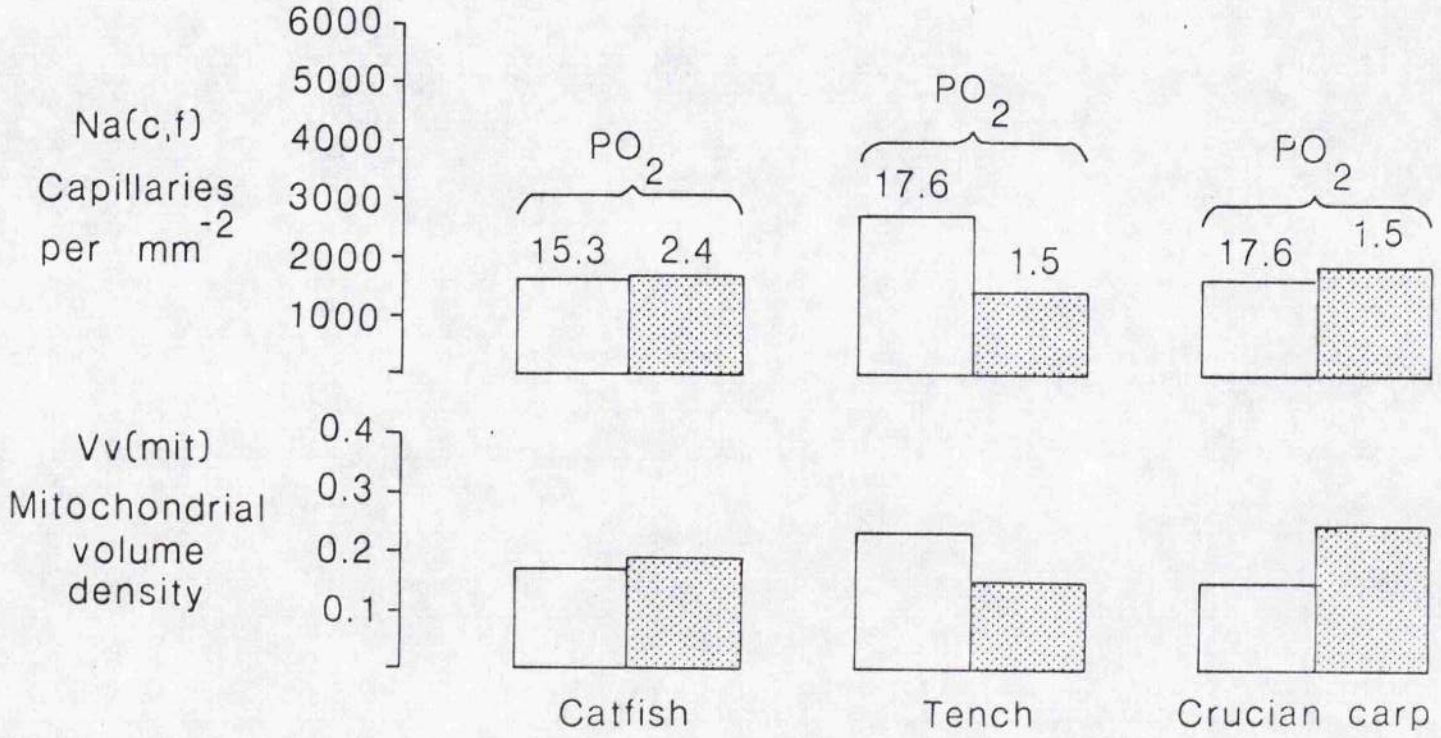

Na(c,f)
Capillaries
per mm-2
6000
5000
4000
3000
2000
1000
PO2
15.3
2.4
PO2
17.6
1.5
PO2
17.6
1.5
Vv(mit)
Mitochondrial
volume
density
0.4
0.3
0.2
0.1
Catfish
Tench
Crucian carp

Figure 6:8

Histograms showing the effects of acclimation to hypoxic water on the mitochondrial volume density ($V_{v\ (mit)}$) and capillary density ($N_{a\ (c,\ f)}$) of the fast glycolytic muscles of the tench, Crucian carp and catfish. The figures above each bar represent the P_{O_2} at which the fish were acclimated.

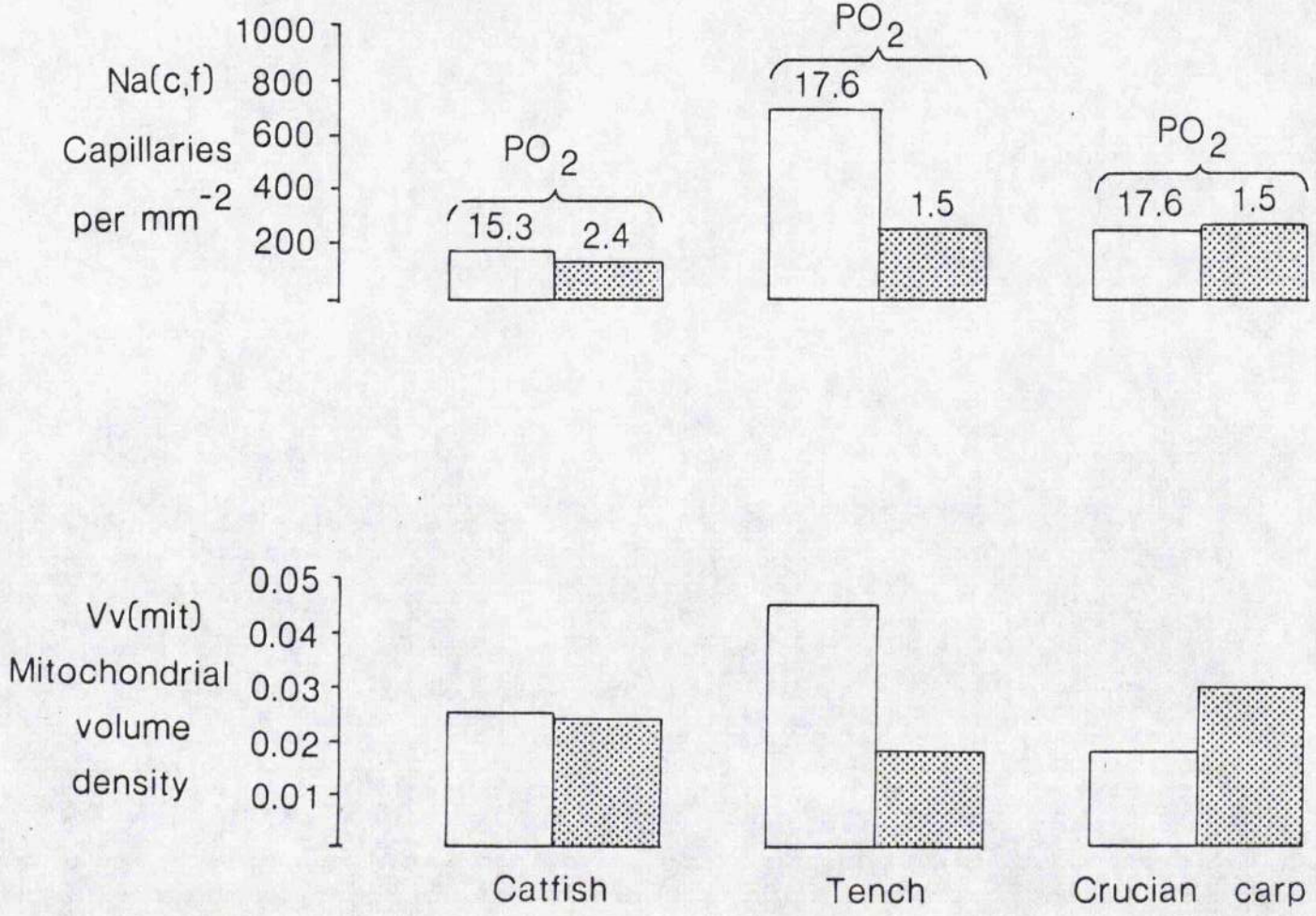

Na(c,f)
Capillaries
per mm-2
1000
800
600
400
200
PO2
15.3
2.4
PO2
17.6
1.5
PO2
17.6
1.5
Vv(mit)
Mitochondrial
volume
density
0.05
0.04
0.03
0.02
0.01
Catfish
Tench
Crucian carp

Figure 6:9

Histogram showing the frequency distribution of the cross-sectional area of capillaries from the myotomal muscle of the juvenile eel (Anguilla anguilla). From Egginton, 1982. Note that the cross-sectional area of most of the fibres lies very close to the mean.

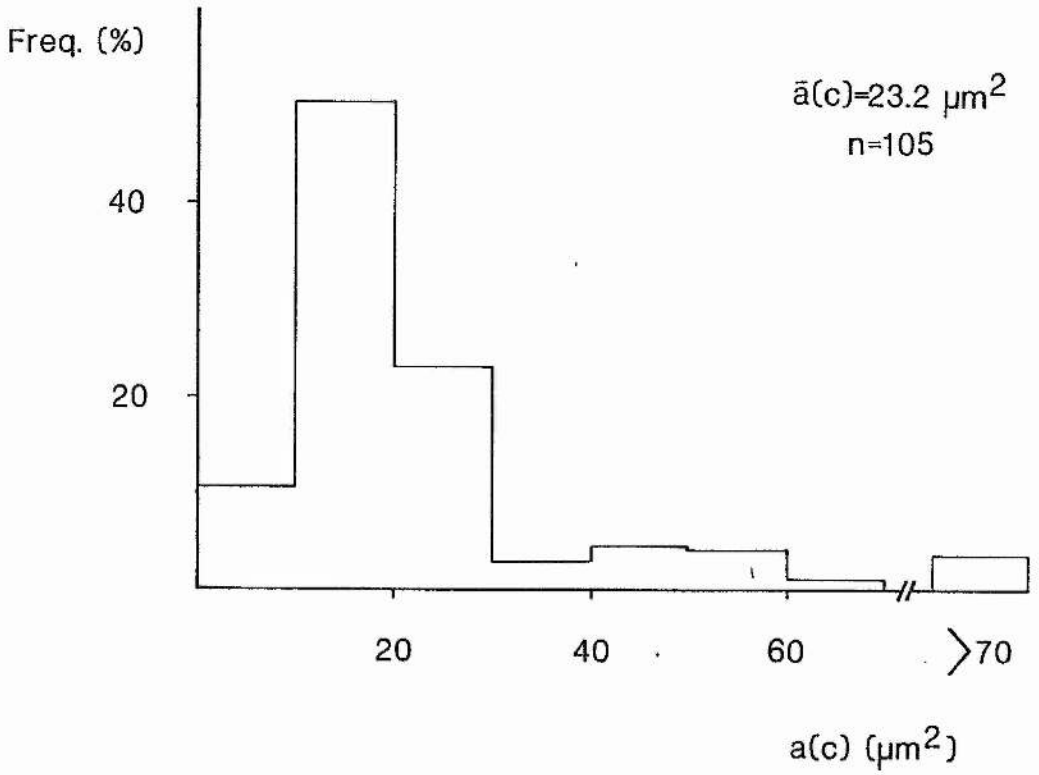

Freq. (%)
40
20
ā(c)=23.2 µm²
n=105
20
40
60
>70
a(c) (µm²)

Figure 6:10

The fine structure of different muscle fibres in the Crucian carp, Carassius carassius, myotome. A series of transverse sections of muscle from fish acclimated to aerated water.

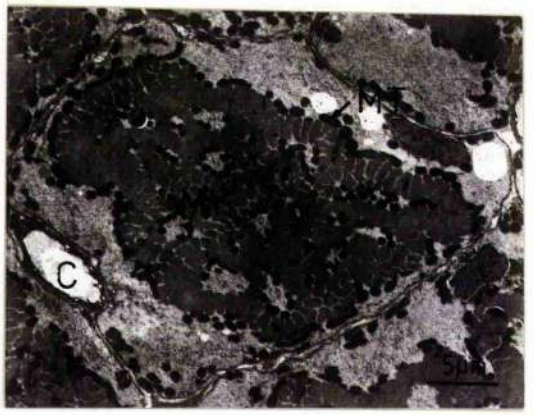
(a) Slow fibre from the lateral line triangle. MT mitochondria; MY myofibrils; C capillary.

(b) Small diameter fast glycolytic fibre. Note the ribbon like peripheral myofibrils and sparse mitochondria. MT mitochondria; MY myofibrils.

(c) Low power micrograph of slow fibres and their associated capillaries (C). This is the type of area analysed to assess capillary supply.

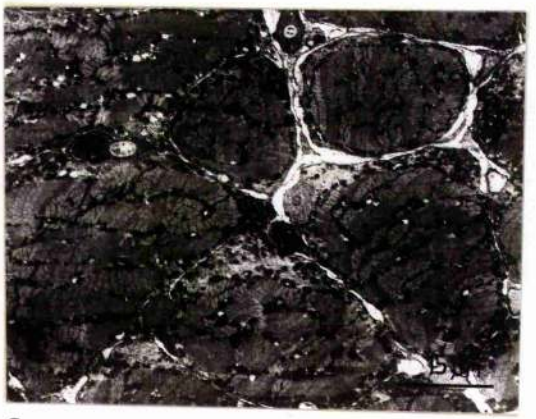
(d) Typical mitochondria from a slow fibre.

(e) Typical mitochondria from a slow fibre.

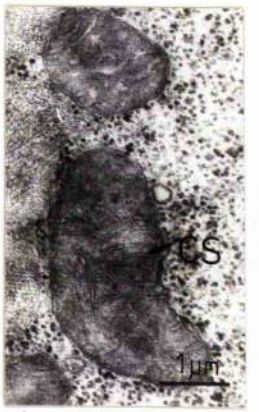


a

b



c

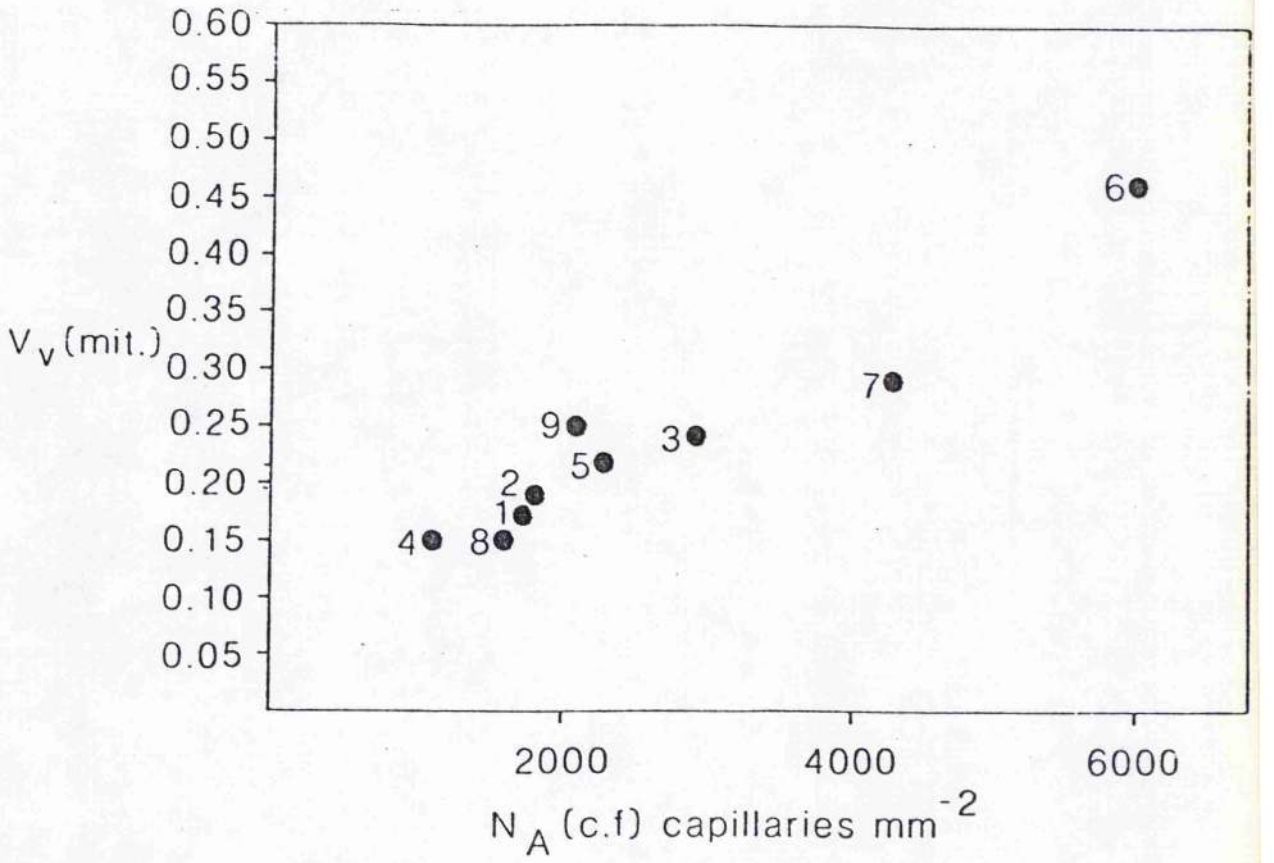


d

e

Figure 6:11

Relationship between mitochondrial volume density ($V_{v\ (mit)}$) and the number of capillaries per mm^2 of muscle fibre cross-sectional area $N_{A\ (c,\ f)}$ for fish slow fibres. (1) Catfish (Clarias mossambicus) acclimated to aerated water, (2) Catfish (Clarias mossambicus) acclimated to hypoxic water, (3) Tench (Tinca tinca) acclimated to aerated water, (4) Tench (Tinca tinca) acclimated to hypoxic water, (5) Elver (Anguilla anguilla) (Egginton & Johnston, 1982 a, b) (6) European anchovy (Engraulis encrasicolus), (Johnston, 1982 c), (7) Silver Dollar (Myleus rosevetti) acclimated to 24°C in aerated water (Salamonski & Johnston, unpublished results), (8) Crucian carp (Carassius carassius) acclimated to aerated water, (9) Crucian carp (Carassius carassius) acclimated to hypoxic water.


0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
V_V (mit.)
6
7
9
3
5
2
1
4
8
2000
4000
6000
N_A (c.f) capillaries mm^{-2}

CHAPTER 7

DISCUSSION

Various studies have established that the ultrastructural characteristics of fish muscle are not fixed but subject to variation with changes in activity patterns and environmental variables. It was previously known that factors such as temperature change could induce changes in muscle capillary supply. However, no study had considered the effects of hypoxia acclimation on these parameters. Johnston and Maitland (1980) have studied the effects of acclimation to temperatures of 2°C and 28°C on the red and white muscles of the Crucian carp, Carassius carassius. Mitochondrial volume fraction, $V_{v\ (m,f)}$, increased in both fibre types following cold acclimation from 14.7% at 28°C to 31.4% at 2°C in the slow muscles and from 1.6% at 28°C to 6.1% at 2°C in the fast muscles.

There appears to be a clear relationship between capillary density, $(N_{A\ (c,f)})$, and mitochondrial volume density, $(V_{v\ (m,f)})$, in the slow muscles of various species studied (Fig. 6:11, Chap. 6). This correlation might imply that one of the limiting factors in determining the size of the mitochondrial compartment and hence the aerobic capacity, is the capillary network available for gas exchange. Hoppeler et al, (1981 a,b), have demonstrated a similar, though more scattered, relationship between $N_{A\ (c,f)}$ and $V_{v\ (m,f)}$ for the mixed fibre type muscles of various species of African mammals. Recently, however, a few species have been studied which do not fit into this general relationship. The Conger eel, Conger conger, has a capillary density, $N_{A\ (c,f)}$ of 615 and $V_{v\ (m,f)}$ of 0.23 (Egginton & Johnston 1983b) and the haemoglobinless icefish, Chaenocephalus aceratus, has an $N_{A\ (c,f)}$ of 544 and $V_{v\ (m,f)}$ of 0.30 (Fitch & Johnston, 1983). These fish may be able to modify other factors such as respiration

efficiency or arterial PO_2 or it may be that $N_{A\ (c,f)}$ simply does not provide an adequate assessment of the capillary bed available for gas exchange in these species. The icefish, Chaenocephalus aceratus, is rather an exceptional teleost. Apart from possessing no haemoglobin, Fitch and Johnston (1983), have reported that the average capillary cross-sectional area is 2-3 times greater than for other teleosts which are normally in the range 15-30 um^2 cross-sectional area, (Egginton & Johnston, 1983b). The large size of the muscle capillaries is consistent with reports of large blood volume and low systemic blood pressure found in Notothenoid fishes (Twelves, 1972).

The mitochondrial volume density found in fish slow fibres is very high compared to that found in mammalian aerobic fibres (Johnston, 1981). Slow muscle in fish receives a high proportion of cardiac output during activity (Daxboeck, Randall & Jones, 1982). This would indicate a very aerobic type metabolism. However, the aerobic scope for activity is much lower in the fishes than in mammals and birds (Bennet, 1978). $V_{v\ (m,f)}$ in the finch heart, a highly aerobic muscle is 37% (Bossun, Sommer & Waugh, 1978) which is similar to that found in the slow fibres of many fishes. The $V_{v\ (m,f)}$ of mammalian skeletal muscle is generally much lower, for example the guinea pig soleus has a mitochondrial volume density of 4.8% (Eisenberg et al, 1979). Maximal oxygen consumption in poikilothermic vertebrates is approximately ten times the resting level. This barely approximates to the resting oxygen consumption of a homeotherm. (Fig. 7:1) Birds and mammals have much greater capacities for sustained, aerobically supported activity whereas fish and other poikilotherms rely on anaerobic metabolism to support sudden or intense activity (Bennet, 1978). The precise reason for the difference in aerobic capacities between poikilotherms and homeotherms is not known. The amounts of slow and fast muscle present in the body is an important factor. However this

is difficult to assess in birds and mammals due to the mosaic type arrangement of the muscle fibre types. ATP turnover rates may be slower at the lower body temperatures found in the poikilotherms. Maximum aerobic capacity is ultimately determined by the volume density of mitochondria and the activity and number of respiratory chains on the inner mitochondrial membrane coupled with the capacity of the capillary circulation to deliver oxygen and substrates. There is little known about differences in mitochondrial activity and respiratory chains between homeotherms and poikilotherms.

Muscle, unlike many other tissues, has been shown to be a very "plastic" tissue, not only on the gross scale, hypertrophing and atrophying in response to various stimuli, but also at the ultra-structural level with changes in the sizes of the compartments occupied by the various cell organelles. Exercise training in mammals has been shown to induce two types of adaptive response in skeletal muscle depending on the nature of the exercise. Exercise such as weight training in man induces muscle cell hypertrophy and an increase in strength whereas endurance exercise such as long distance running induces an increase in the muscles capacity for aerobic metabolism (Holloszy and Booth, 1976). Both the size and number of mitochondria increase in response to regular training in both the rat and humans. (Gollnick & Lanuzzo, 1972; Hoppeler et al, 1973; Morgan et al, 1969) However, it appears from the few studies undertaken that the responses of mammals and fish to endurance training are very different and probably related to the lower aerobic scope for activity found in fish (Bennet, 1978). Johnston and Moon (1980) have shown that exercise training in the saithe, Pollochius virens, led to a large increase in red and white muscle creatine kinase activities but little change in the activities of the tricarboxylic acid cycle and respiratory chain enzymes suggesting that increased energy production in the exercise

trained fish must be met anaerobically. Starvation caused muscle atrophy and a fall in mitochondrial volume fraction from 34.6 to 18.6% in the saithe, (Beardall & Johnston, 1983). This study has shown that hypoxia acclimation is another factor which influences the ultrastructure of skeletal muscle.

An important point to consider is the fact that the method used to examine capillaries in this study only looks at them in a two dimensional plane. Obviously capillaries are three dimensional entities. Recently Hoppeler et al, (1981), and Mathieu et al, (1981) have developed methods for determining capillary anisotropy. Calculations of capillary volume density, $J_{v\ (c,f)}$ and surface density, $S_{v\ (c,f)}$ take into account the length of capillaries relative to the volume of muscle fibres and provide an estimate of the volume of capillary blood and available surface area for gas/metabolite exchange respectively. These are more physiologically relevant parameters than numerical capillary density. Egginton and Johnston, (1983b) have shown, however, for the conger eel that there is a remarkable degree of anisotropy in the slow muscle capillary network, so much so that the capillary density $N_{A\ (c,f)}$ provides a reasonable estimate of $J_{v\ (c,f)}$ provided that the sample size is large enough and true transverse sections are used.

Wolff et al (1975) have suggested that capillary density will increase along the length of a muscle to compensate for the reduced P_{O_2} at the venule end. However there is no information available concerning capillary and mitochondrial distribution along the length of muscle fibres in fish to support this theory. The new types of analyses mentioned could present exciting areas for future research on fish muscle and the effects of environmental variables on its structure and function.

The strict definition of hypoxia refers to a lack of oxygen at the cellular level. There is a gradient of oxygen tensions

along the respiratory chain (Fig. 7:2) and oxygen passing from the environment must meet a series of resistances during its transfer to the cell. (Hughes, 1973). Should this resistance increase at any one of the levels shown in Fig. 7:2 hypoxia would result at the cellular level. However, at each of these levels there is also potential for the animal to compensate by adaptation to the reduced oxygen level.

Each level may be considered separately and possible methods of adaptation summarised.

A. The P_{O_2} of water in different habitats is subject to enormous variation as has already been mentioned in Chapter 1. Hypoxia in a fishes environment might arise due to diurnal variation with plant photosynthesis, over-population of the environment, pollution, temperature, depth or eutrophication plus a variety of other causal factors. A fall in the oxygen level of the environment will primarily affect the driving force into the gills and thus is likely to modify oxygen levels along the whole chain. The only method a fish has of altering environmental oxygen level is by it behaviour. Most species will evade hypoxic water. However, this is not always possible so alternative strategies of compensation must be adopted.

B. The ventilation of the respiratory cavities has a bearing on the amount of oxygen containing water which comes into contact with the gill surfaces. Hypoventilation occurs in rare cases but would result in oxygen depletion and CO_2 build-up in the water in the gill cavities. One strategy to increase oxygen transfer into the gills would be to increase the amount of water flowing over the gills by hyperventilating. However the amount of oxygen gained in this way must be balanced with the extra amount needed to propel the muscles involved in moving the respiratory apparatus to pump more water through.

C. A very large resistance is met on reaching the gill lamellae. Pollutants can increase the thickness of the barrier to diffusion

exchange across the gill surfaces thus hindering effective diffusion. The convection of the water current between the secondary lamellae is important and must be arranged to ensure optimum oxygen transfer. By the time oxygen has diffused through the gill membranes it has fallen from around 135mm Hg (at normal environmental oxygen levels) to approximately 80mm Hg. From here it must diffuse through the plasma and red cell membranes before binding with haemoglobin (provided this is present). Several factors can influence this passage. The blood flow rate through the gill capillaries and its P_{O_2} will determine available time for transfer and the size of the diffusion gradient. The Bohr effect in which CO_2 in the blood reduces the affinity of haemoglobin for oxygen facilitates loading in the gills and unloading in the tissues. Fish show varying Bohr, Root and Haldane effects in their blood depending on their habitat and way of life. Fish from habitats with lower oxygen levels tend to have low P_{50}s and the Bohr effect does not put the $Hb\text{-}O_2$ dissociation curve out of the useful range (Prosser, 1973). Fish blood is also very sensitive to temperature rises and to pollutants which can cause anaemia through binding of the functional haemoglobin and some of which can affect the shape of the red cells thus preventing them from entering the capillaries. (Hughes, 1973). The effects of the concentrations of the nucleoside triphosphates on $Hb\text{-}O_2$ binding have already been discussed in Chapter 1. Wood and Johansen (1972) have shown that acclimation to hypoxia in eels causes a shift in the blood - O_2 dissociation curve to the left and a reduction in the ATP content of the red cells. The increased ATP concentration in the red cells changes the properties of the haemoglobin so that there is an increase in oxygen carrying capacity and affinity which favours loading at the gills in hypoxic water.

The final part of the respiratory chain involving the passage of oxygen from the blood cells in the capillaries (G, H, I) to the

mitochondria is the part which this study is mainly concerned with. Little is known about the resistances met by oxygen molecules at this level. Some of the main modifiers are illustrated in Fig. 7:3. The P_{O_2} of the blood in the capillaries will affect the size of the diffusion gradient into the muscle cells. Blood P_{O_2} is likely to fall as the blood moves from the arterial to the venular end of the capillary bed thus the diffusion gradient will be smaller at the venule end. There may be compensatory methods of dealing with this in the tissue. However there is little information available on this subject. Weibel (1981) has suggested that capillary density increases towards the venular end in mammals. The flow rate of the blood through the capillaries will determine the time available for oxygen transfer to the cells. The Bohr and Root effects facilitate unloading of oxygen at the tissues and the altered Bohr and Root effects seen after acclimation to hypoxia in several species will help compensate for the lack of oxygen in the blood by unloading more of the oxygen bound to haemoglobin (Prosser, 1973). Fish show an enormous variety of effect of CO_2 and temperature on oxygen affinity so no general conclusions can be drawn until more detailed information is available for many species (Prosser, 1973).

Muscle myoglobin can transfer oxygen from blood to cell enzymes due to its greater affinity for O_2 than blood haemoglobin, thus the presence of myoglobin and its concentration will be important in facilitating diffusion into the muscle cells.

Ultimately the oxidative generation of ATP occurs in the mitochondria, on the respiratory chain units packed into the inner mitochondrial membranes (cristae). The position of a mitochondrian within a muscle cell will determine to a large extent how much oxygen it receives. Subsarcollemmal mitochondria lying in close proximity to a capillary will receive more oxygen than interfibrillar mitochondria

due to the reduced diffusion distance. Weibel et al (1981) have shown that mitochondria are not evenly distributed throughout muscle fibres in several species of African mammal but are concentrated around the surface areas close to the capillaries. The number of mitochondria present and their volume density are of importance as are the number of respiratory chains present and the surface densities of the outer and inner membranes. There is no information available on the latter factors in fish.

From a consideration of this respiratory chain from environment to mitochondria it is clear that there are numerous strategies which a fish may adopt to maintain respiratory homeostasis when it experiences hypoxia in its environment. It is obvious therefore that not every species will adapt in the same way to the same stimulus. The three species of fish examined in this study have shown different adaptation methods and it must be remembered that only a few points were looked at so the adaptations seen were probably combined with others not yet studied.

The tench, Tinca tinca, showed adaptations at the behavioral level by a distinct lowering of activity. The hypoxia acclimated animals moved around the tank much less that the normoxia acclimated animals. Oxygen consumption fell from 32.7 mls O_2/Kg/h in aerated water to 10.8 mls O_2/Kg/h on acute exposure to hypoxia. However after 6 weeks acclimation to hypoxia oxygen consumption was 15.6 mls O_2/Kg/h, a 17% increase. Both mitochondrial volume density and capillary density decreased. This would be in keeping with the reduction in activity and hibernation in the wild but some other, as yet unexamined, point on the respiratory chain must have been modified to account for the increased oxygen extraction capacity of hypoxia acclimated fish.

The Crucian carp, Carassius carassius, also increased its oxygen extraction capacity. Routine oxygen consumption in aerated water was 75.7 mls O_2/Kg/h. At P_{O_2} 1.5 KPa. oxygen consumption in normoxia acclimated animals was 16.2 mls O_2/Kg/h whereas that of hypoxia acclimated animals was 34 mls O_2/Kg/h. Capillary density was little affected by hypoxia acclimation but a significant (67%) increase in slow muscle mitochondrial volume density was found. It would appear that this increase in $V_{v\ (m,f)}$ might represent an adaptation to increase the utilisation of circulating oxygen stores. The increased O_2 extraction by the muscles would reduce venous P_{O_2} and therefore increase the rate of oxygen transfer across the gills. It is likely that alterations in other parameters have occurred such as the haemoglobin properties, blood flow and the number of perfused capillaries and these alterations have proved sufficient to meet the routine oxygen needs without changes in the capillary bed being necessary.

The air breathing catfish, Clarias mossambicus, through a combination of its air breathing ability and the oxygen obtained through the gills was able to increase its total oxygen extraction after acclimation to hypoxia. Routine respiration rate for fish acclimated to aerated water was 85.7 mls O_2/Kg/h. On acute exposure to hypoxia this fell to 46.3 mls O_2/Kg/h. However after acclimation to hypoxia it rose to 67.8 mls O_2/Kg/h largely as the result of an increase in aquatic respiration from 10.4 to 27.5 mls O_2/Kg/h.

Mitochondrial volume density and capillary density were altered very little by hypoxia acclimation suggesting that the increased oxygen extraction through the suprabranchial organs and at the gills obviates the need for any modification of these parameters. It is likely that changes in the haeomoglobin binding properties have occurred to increase oxygen extraction, especially across the gills, but this has yet to be examined.

Each of the species studied appears to have adapted to the hypoxic stress in a different way. In view of the number of points on the pathway of oxygen from environment to mitochondria which are subject to possible modification it would be perfectly reasonable to expect different species to adapt in different ways. Little is known about the mechanisms responsible for changes in capillary density and the size of the mitochondrial compartment with different physiological states. It would appear that they are determined by a complex interplay of both physical and chemical factors.

Only the responses of a few tissues and parameters and indeed species have been examined in this study. Before a complete understanding of the mechanisms of hypoxia adaptation could be achieved it would be necessary to examine other facets and many more species. Studies of such factors as blood/O_2 extraction capacities, gill function and blood perfusion conducted in parallel with the type of measurements made in this study, ie capillary supply, mitochondrial volume density and oxygen consumption, plus further measurements of enzyme activities, metabolite concentrations, active oxygen consumptions and further extrapolation of the structural parameters to $J_{v\ (c,f)}$ and $S_{v\ (c,f)}$ would give a more complete assessment of the responses of fish to chronic hypoxia. There is obviously enormous scope for further research into this complex subject of hypoxia acclimation.

Figure 7:1 Graph showing rates of oxygen consumption plotted against body mass for a variety of animals (log coordinates). The points fall along regression lines with slopes of 0.75. Each division on the coordinates signifies a 1000-fold change. (After Hemmingsen, 1960.)

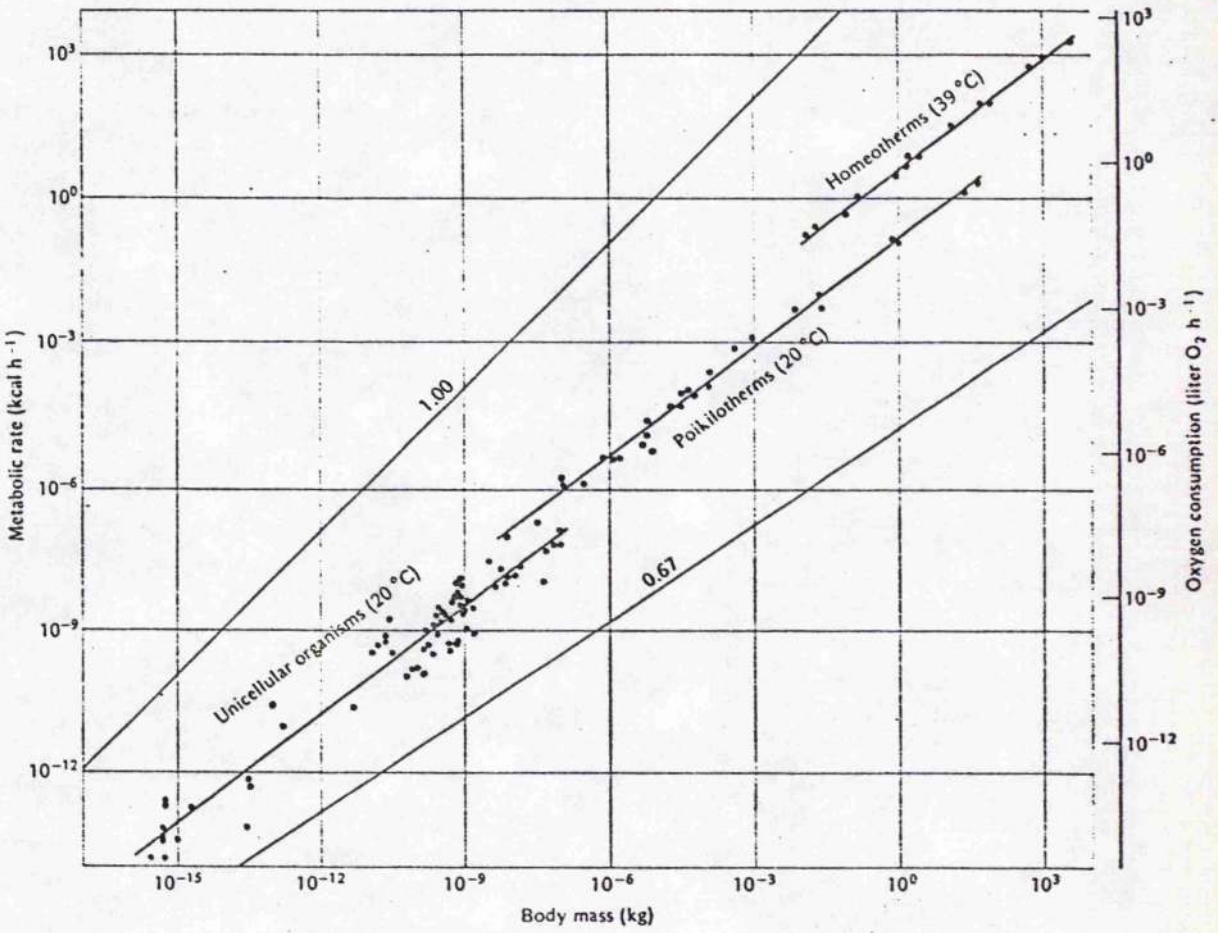

Metabolic rate (kcal h⁻¹)
Oxygen consumption (liter O₂ h⁻¹)
Body mass (kg)
Homeotherms (39 °C)
Poikilotherms (20 °C)
Unicellular organisms (20 °C)
1.00
0.67
10³
10⁰
10⁻³
10⁻⁶
10⁻⁹
10⁻¹²
10⁻¹⁵

Figure 7:2 Diagram indicating changes in oxygen tension along the respiratory chain of fish from the external water (P_{in}) to the mitochondria (P_{mit}). Some of the main resistances are shown as is a qualitative indication of the relative drop across them (e.g. P_{il} and P_m). Parts of the chain mainly affected by different types of hypoxia (A - I) mentioned in Chapter 7 are indicated. (After Hughes, 1973.)

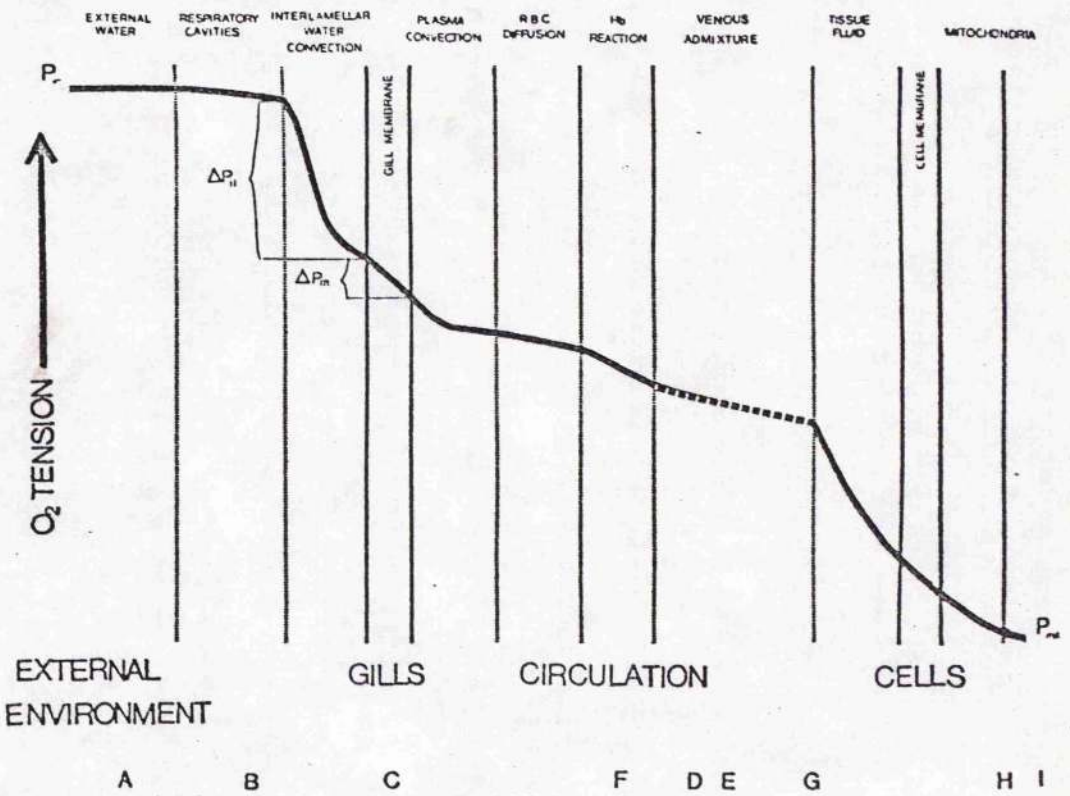
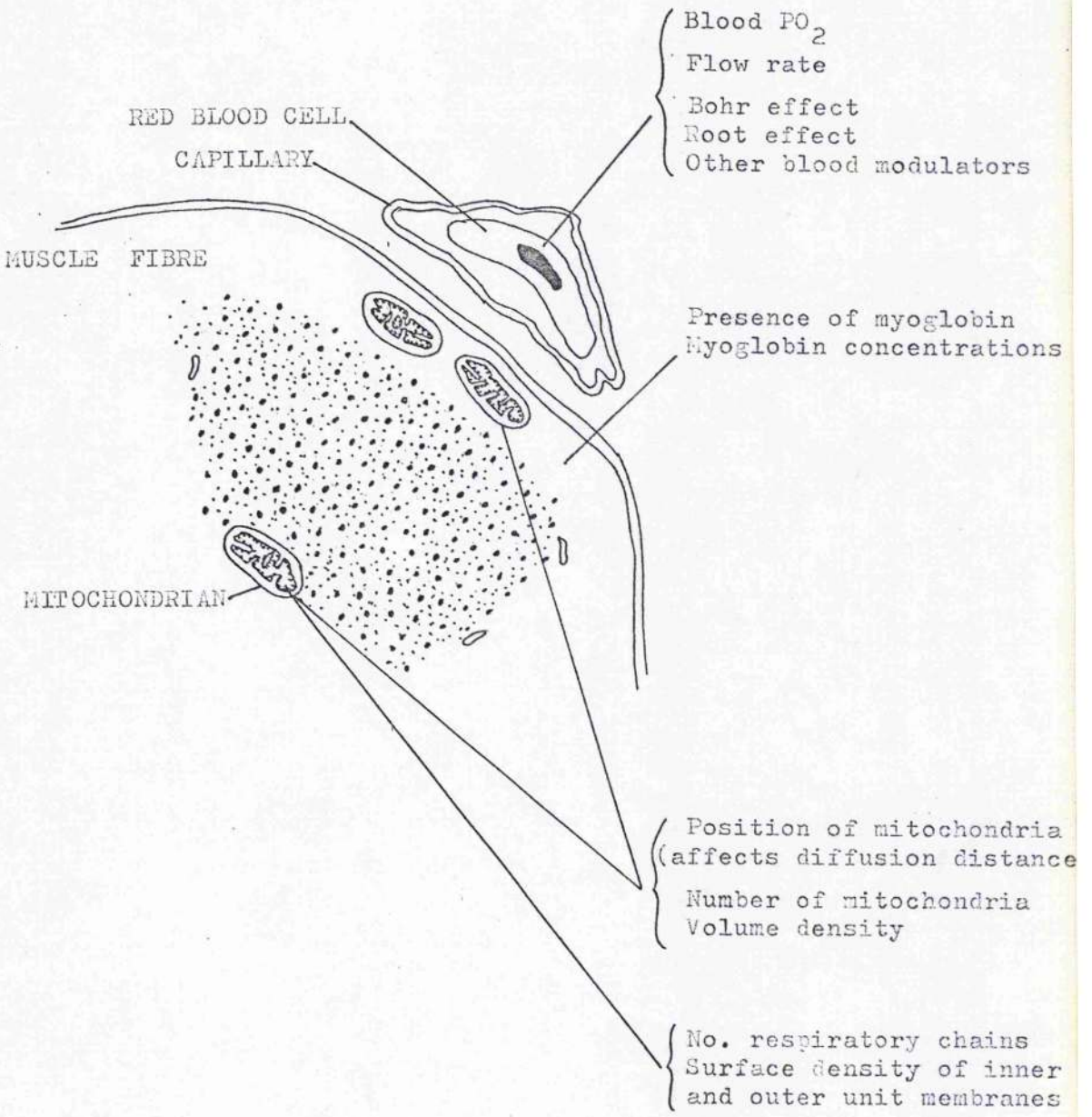

EXTERNAL WATER
RESPIRATORY CAVITIES
INTERLAMELLAR WATER CONVECTION
PLASMA CONVECTION
RBC DIFFUSION
Hb REACTION
VENOUS ADMIXTURE
TISSUE FLUID
MITOCHONDRIA
GILL MEMBRANE
CELL MEMBRANE
P_e
ΔP_{il}
ΔP_{m}
P_{mi}
O_2 TENSION
EXTERNAL ENVIRONMENT
GILLS
CIRCULATION
CELLS
A
B
C
F
D
E
G
H
I

Figure 7:3 Diagram illustrating some of the main modifiers affecting the passage of oxygen from the capillaries to the mitochondria.

MODULATORS


Blood PO_2
Flow rate
Bohr effect
Root effect
Other blood modulators
RED BLOOD CELL
CAPILLARY
MUSCLE FIBRE
Presence of myoglobin
Myoglobin concentrations
MITOCHONDRIAN
Position of mitochondria
(affects diffusion distance
Number of mitochondria
Volume density
No. respiratory chains
Surface density of inner
and outer unit membranes

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REFERENCES

Akster H.A. (1981). Ultrastructure of muscle fibres in head and axial muscles of the perch (Perca fluviatilis L.). Cell Tiss. Res. 219 : 111-131

Alexander R. McN. (1969). The orientation of muscle fibres in the myomeres of fishes. J.mar.biol. Ass. UK. 49 : 263-290

Altringham J.D. & Johnston I.A. (1981a). Quantitative histochemical studies of the peripheral innervation of cod (Gadus morhua) fast myotomal muscle fibres. J. Comp. Physio. 143 : 123-127

Altringham J.D. & Johnston I.A. (1981b). Force velocity relationship of skinned fibres isolated from dogfish myotomal muscle. J. Physiol. 319 : 85-86.

Altringham J.D. & Johnston I.A. (1983). The pCa-tension and force-velocity characteristics of skinned fibres isolated from fish fast and slow muscles. J. Physiol. 333 : 421-429

Banchero N, Gimenez M, Rostami A & Eby S.H. (1976) Effects of simulated altitude on O_2 transport in dogs. Respir. Physiol. 27 : 305-321

Bárány M. (1967). ATPase activity of myosin correlated with speed of muscle shortening. J. gen. Physiol. 51 : 197-216

Barets A. (1961). Contribution a l'etude des systemes moteur lent et rapide du muscle lateral des teleosteens. Archs. Anat. Morph. exp. 50 (suppl) : 91-187

Beamish F.W.H. (1964). Respiration of fishes with special emphasis on standard oxygen consumption. III Influence of oxygen. Can. J. Zool. 42 : 355-366

Beardall C.H. & Johnston I.A. (1983). Muscle atrophy during starvation in a marine teleost. European J. Cell Biol. 29 : 209-217

Bennet A.F. (1978). Activity metabolism of the lower vertebrates. An. Rev. Physiol. 40 : 447-469

Best A.C.G. & Bone Q. (1973) The terminal neuro-muscular junctions of lower chordates. Z. Zellforsch. Mikrosk. Anat. 143 : 495-504

Bilinski E. (1974). Biochemical aspects of fish swimming. In 'Biochemical Perspectives in Marine Biology. ed. D.C. Maling & J.R. Sargent, Vol. 1, pp 239-288. New York : Academic Press.

Bilinski E. & Jonas R.E.E. (1972). Oxidation of lactate to carbon dioxide by rainbow trout (Salmo gairdneri) tissues. J. Fish Res. Bd. Can. 29 : 1467-1471

Black E.C., Robertson A.C. & Parker R.R. (1961). Some aspects of carbohydrate metabolism in fish. In 'Comparative Physiology of carbohydrate metabolism in heterothermic animals. pp 89-124. Ed. A.W. Martin, Seattle : Univ. Washington.

Blâzka P. (1958). The anaerobic metabolism of fish. Physiol. Zool. 31 : 117-128

Bone Q. (1964). Patterns of muscular innervation in the lower chordates. Int. Rev. Neurobiol. 6 : 99-147

Bone Q. (1966). On the function of the two types of myotomal muscle fibres in elasmobranch fish. J. mar. biol. Ass. UK. 46 : 321-349

Bone Q. (1970). Muscular innervation and fish classification. Simp. Int. Zoofil. 1st Univ. Salamanca 1970 : 369-377

Bone Q. (1972). The dogfish neuromuscular junction : Dual innervation of vertebrate striated muscle fibres? J. Cell Sci. 10 : 657-665

Bone Q. (1978a). Locomotor muscle. In 'Fish Physiology' Vol 7. pp 361-424. Hoar W.S. & Randall D.J. (Eds) New York & London. Academic Press.

Bone Q. (1978b). Myotomal muscle fibre types in Scomber and Katsuwonus. In 'The physiological ecology of tunas' pp 183-205 Sharp G.D. & Dizon A.E. (Eds). New York & London : Academic Press

Bone Q. & Chubb A.D. (1978). The histochemical demonstration of myofibrillar ATPase in elasmobranch muscle. Histochem. J. 10 : 489-494

Bone Q. & Johnston I.A. (1982). Contractile properties of dogfish muscle fibres. J. mar. biol. Ass. UK. in press.

Bone Q., Kicznuik J. & Jones D.R. (1978). On the role of the different fibre types in fish myotomes at intermediate swimming speeds. Fish. Bull. US. 76 : 691-699

Bone Q. & Ono R. (1982). Systematic implications of innervation patterns in teleost myotomes. Breviora. 470 : 1-23

Bossen E.J., Sommer J.R. & Waugh R.A. (1978). Comparative stereology of the mouse and finch left ventricle. Tissue & Cell. 10 : 773-784

Boström S.L. & Johansson R.G. (1972). Enzyme activity patterns in white and red muscle in the eel (Anguilla anguilla) at different developmental stages. Comp. Biochem. and Physiol. 42B : 533-542

Brett J.R. (1972). The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. Respiration Physiology. 14 : 151-170

Brill R.W. & Dizon A.E. (1979). Red and white muscle fibre activity in swimming skipjack tuna, Katsuwonus pelamis L. J. Fish Biol. 15 : 679-685

Brown M.D., Cotter M.A., Hudlicka O. & Vrbora G. (1976). The effect of different patterns of muscle activity on capillary density, mechanical properties and structure of slow and fast rabbit muscles. Pflugers Arch. 361 : 241-250

Burton D. & Spehar A. (1971). A re-evaluation of the anaerobic end-products of fresh-water fish exposed to environmental hypoxia. Comp. Biochem. Physiol. 40A : 945-954

Cech J.J., Bridges D.W., Rowell D.M. & Balzer P.J. (1977). Cardio-vascular responses of winter flounder to acute temperature increase. Can. J. Zool. 54 : 1383-1388

Collins J.H. (1976). Structure and evolution of troponin C and related proteins. Symp. Soc. exp. Biol. 9 : 303-334

Coulter G.W. (1967). Low apparant oxygen requirements of deep-water fishes in Lake Tanganika. Nature 215 : 317-318

Crabtree B. & Newsholme E.A. (1972). The activities of phosphorylase, hexokinase, phosphofructokinase, lactate dehydrogenase in muscles from vertebrates and invertebrates. Biochemical Journal. 126 : 49-58

Daxboeck C., Randall D.J. & Jones D.R. (1982). Cardiac output redistribution during swimming exercise in a teleost fish, Salmo gairdneri J. exp. Biol. in press

Driedzic W.R. & Hochachka P.W. (1978). Metabolism of fish during exercise. In 'Fish Physiology' vol 7 : pp 503-543. Eds. W.S. Hoar & D.J. Randall. New York & London : Academic Press

Eby S.H. & Banchero N. (1976). Capillary density of skeletal muscle in Andean dogs. Proc. Soc. Exp. Biol. Med. 151 : 795-798

Egginton S. & Johnston I.A. (1982a). A morphometric analysis of regional differences in myotomal muscle ultrastructure in the juvenile eel, Anguilla anguilla, L. Cell Tiss. Res. 222 : 579-596

Egginton S. & Johnston I.A. (1982b). Muscle fibre differentiation and vascularisation in the juvenile European eel (Anguilla anguilla) Cell Tiss. Res. 222 : 563-577

Egginton S. & Johnston I.A. (1983a). Suitability of measured parameters and minimum sample sizes required to quantify capillary supply to fish muscle. Personal communication.

Egginton S. & Johnston I.A. (1983b). An estimate of capillary anisotropy and determination of surface and volume densities of capillaries in skeletal muscles of the Conger eel (Conger conger L.). Quart. J. Exp. Physiol. 68 : 603-617

Eisenberg B.R., Kuda A.M. & Peter J.B. (1974). Stereological analysis of mammalian skeletal muscle. J. Cell. Biol. 60 : 732-754

Fish G.R. (1956). Some aspects of the respiration of six species of fish from Uganda. J. exp. Biol. 33 : 186-193

Fitch N.A. & Johnston I.A. (1983). Muscle capillary supply in an Antarctic fish (Chaenocephalus aceratus) which lacks respiratory pigments. J. Physiol. 340 : 65P

Flitney F.W. & Johnston I.A. (1979). Mechanical properties of isolated fish red and white muscle fibres. J. Physiol. Lond. 295 : 49-50

Focant B., Huriaux F. & Johnston I.A. (1976). Subunit composition of fish myofibrils : the light chains of myosin. Int. J. Biochem. 7 : 129-133

Franzini-Armstrong C. & Porter K.R. (1964). Sarcolemmal invaginations constituting the T-system in fish muscle fibres. J.Cell.Biol. 22 : 675-696

Franzini-Armstrong C. (1973). Membranous systems in muscle fibres. In 'The Structure and function of muscle'. Ed. G.H. Bourne. Academic Press, New York, Vol II. pp 532-640

Freadman M.A. (1979). Role of partitioning of swimming muscalature of striped Bass, Morone saxitilis, Walbaum and Bluefish, Pomatomus saltatrix L. J. Fish Biol. 15 : 417-473

Fry F.E.J. (1947). Effects of the environment on animal activity. Univ. Toronto Studies Biol. Ser. 55 : 1-62

Fujikawa K. & Naganuma H. (1936). Chemical composition of sardine, Sardinia melanosticta (C8V) from Tyosen I. Comparative Study on dark and white muscle. Bull. Jap. Soc. Scient. Fish. 5 : 95-102

Gee J.H. (1980). Respiratory patterns and antipredator responses in the central mud minnow, Umbra limi, a continuous facultative air-breathing fish. Can. J. Zool. 58 : 819-827

Gerday C. (1982). Soluble calcium-binding proteins from fish and invertebrate muscle. Mol. Physiol. 2 : 63-87

Gerday C. & Gillies J.M. (1976). The possible rcle of parvalbumins in the control of contraction. J. Physiol. Lond. 258 : 96-97

Gollnick P.D. & Lanuzzo C.D. (1972). Hormonal deficiencies and the metabolic adaptations of rats to training. Am. J. Physiol. 223 : 278-282

Gollnick P.D. & King D.W. (1969). The immediate and chronic effect of exercise on the number and structure of skeletal muscle mitochondria. In 'Medicime and Sport' Vol. 3. pp 239-244. Eds. Poortmans, Kargel, Basel; New York

Gordon M.S. (1972). Comparative studies on the metabolism of shallow-water and deep-sea marine fishes. II Red-muscle metabolism in shallow-water fishes. Marine Biol. 15 : 246-250

Graham J.B. & Baird T.A. (1982). The transition to air-breathing in fishes. I. Environmental effects on the facultative air-breathing of Ancistrus chagresi and Hypostomus plecostomus (Loricardiidae). J. exp. Biol. 96 : 53-67

Greenwood P.H. (1958). In 'The Fishes of Uganda'. The Uganda Society, Kampala. p.p. 124

Greer-Walker M. (1970). Growth and development of the skeletal muscle fibres of the cod (Gadus morhua L.). J. Cons. perm. int. Explor. Mer. 33 : 228-244

Greer-Walker M. (1971). The effects of starvation and exercise on the skeletal muscles of the cod (Gadus morhua L.) and the coalfish (Gadus virens L.) respectively. J. Cons. int. Explor. Mer. 33 : 421-427

Greer-Walker M. & Pull G.A. (1975). A survey of red and white muscle in marine fish. J. Fish. Biol. 7 : 295-300

Guppy M., Hulbert W.S. and Hochachka P.W. (1979). Metabolic sources of heat and power in tuna muscles. II. Enzyme and metabolite profiles. J. exp. Biol. 82 : 303-320

Hagiwara S. & Takahashi K. (1967). Resting and spike potentials of skeletal muscle fibres in salt-water elasmobranch and teleost fish. J. Physiol. Lond. 190 : 499-518

Hall F.G. (1929). O_2 withdrawal and consumption by marine fish. Amer. J. Physiol. 88 : 212-218

Hazel J. & Prosser C.L. (1974). Molecular mechanisms of temperature compensation in poikilotherms. Physiological Reviews. 84 : 620-677

Heath A.G. & Pritchard A.W. (1965). Effects of severe hypoxia on carbohydrate energy stores and metabolism in two species of freshwater fish. Physiol. Zool. 38 (4) : 325-333

Hemmingsen A.M. (1960). Energy metabolism as related to body size and respiratory surfaces, and its evolution. Rep. Steno Hosp. 9 (2) : 1-110

Hemmingsen E. A. (1965). Accelerated transfer of oxygen through solutions of heme pigments. Acta. physiol. Scand. 64 suppl 246 : 1-53

Heroux O. & St. Pierre J. (1957). Effect of cold acclimation on vascularisation of ears, heart, liver and muscles of white rats. Am. J. Physiol. 188 : 163-168

Hess A. (1970). Vertebrate slow muscle fibres. Physiol. Rev. 50 : 40-62.

Hochachka P.W. (1969). Intermediary metabolism in fishes. In 'Fish Physiology' Vol. 1. Eds. W.S. Hoar and D.J. Randall. Academic press, New York. pp 351-389

Hochachka P.W. (1973). Glucose and acetate metabolism in fish. Can. J. Biochem. 39 : 1937-1941

Hochachka P.W. (1975). Biochemistry at depth. Comp. Biochem. Physiol. (B) 52 : 1-202

Hochachka P.W. (1980). Integrative mechanisms in hypoxia adapted fish. In 'Living without oxygen'. Harvard Univ. Press, 1980 pp 100-117

Holeton G.F. & Randall D.J. (1967). Effect of hypoxia on ventilation and metabolism of rainbow trout, J. Exp. Biol. 46 : 297-305, 317-327

Holloszy J.D. & Booth F.W. (1976). Biochemical adaptations to endurance exercise in muscle. Ann. Rev. Physiol. 38 : 273-291

Hoppelar H., Luthi P., Claassen H., Weibel E.R. & Howald H. (1973). The ultrastructure of normal human skeletal muscle. A morphometric analysis of untrained men, woman and well-trained orienteers. Pfluegers Arch. 344 : 217-232

Hoppeler H., Mathieu O., Krauer R., Classen J., Armstrong R.B. & Weibel E.R. (1981). Design of the mammalian respiratory system. VI. Distribution of mitochondria and capillaries in various muscles. Resp. Physiol. 44 : 87-11

Hoppeler H., Mathieu O., Weibel E.R., Krauer R., Lindstedt S.L. & Taylor C.R. (1981). Design of the mammalian respiratory system. VIII. Capillaries in skeletal muscles. Resp. Physiol. 44 : 129 - 150

Hudlicka O., Pette D. & Staudte H. (1973). The relation between blood flow and enzymatic activities in slow and fast muscles during development. Pflugers Arch. 343 : 341-356

Hudson R.C.L. (1969). Polyneuronal innervation of the fast muscles of the marine teleost, Cottus scorpius. L. J. Exp. Biol. 50 : 47-67

Hudson R.C.L. (1973). On the function of the white muscles in teleosts at intermediate swimming speeds. J. Exp. Biol. 58 : 509-522

Hughes G.M. (1973). Respiratory responses to hypoxia in fish. Am. Zool. 13 : 475-489

Hughes G.M. & Singh B.N. (1971). Respiration in air-breathing fish, the climbing perch, Anabas testudineus (Bloch). Oxygen uptake and carbon dioxide release into air and water. J. exp. Biol. 53 : 265-280

Hughes G.M. & Umezawa S.I. (1968). Respiration of the dragonet and dogfish. J. Exp. Biol. 49 : 557-582

Hulbert W.C. & Moon T.W. (1978). A histochemical, light and electron-microscopic examination of eel, Anguilla rostrata, red and white muscle. J. Fish Biol. 13 : 527-533

Itazawa Y. & Takeda T. (1978). Gas exchange in carp gills in normoxic and hypoxic conditions. Respir. Physiol. 35 : 263-269

James N.T. & Meek G.A. (1979). Stereological analyses of the structure of mitochondria in pigeon skeletal muscle. Cell. Tiss. Res. 202 : 493-503

Johnston I.A. (1974). Studies on the swimming muscalature of the rainbow trout. II. Muscle metabolism during severe hypoxia. J. Fish Biol. 7 : 459-467

Johnston I.A. (1975a). Anaerobic metabolism in the carp (Carassius carassius L.) Comp. Biochem. Physiol. 51B : 235-241

Johnston I.A. (1975b). Pyruvate Kinase from the red skeletal muscalature of the carp (Carassius carassius L.) Biochem. Biophys. Res. Commun. 63 : 115-122

Johnston I.A. (1977). A comparative study of glycolysis in red and white muscles of trout (Salmo gairdneri) and mirror carp (Cyprinus carpio). J. Fish Biol. 11 : 575-588

Johnston I.A. (1980a). Contractile properties of fish fast muscle fibres. Marine Biol. Letters 1 : 323-328

Johnston I.A. (1980b). Specializations of fish muscle. In 'Development and specializations of muscle' pp 123-148 (Soc. exp. Biol. Seminar series, 7) Ed. D.F. Goldspink. Cambridge University Press.

Johnston I.A. (1981a). Structure and function of fish muscles. Symp. Zool. Soc. Lond. 48 : 71-113

Johnston I.A. (1981b). Quantitative analysis of muscle breakdown during starvation in the marine flatfish, Pleuronectes platessa. Cell and Tissue Res. 214 : 369-386

Johnston I.A. (1982a). Capillarisation, oxygen diffusion distances and mitochondrial content of carp muscles following acclimation to summer and winter temperatures. Cell and Tissue Res. 222 : 325-337

Johnston I.A. (1982b). Biochemistry of fish myosins and contractile properties of fish skeletal muscles. J. mol. Physiol. 2 : 15-29

Johnston I.A. & Bernard L.M. (1982a). Routine oxygen consumption and characteristics of the myotomal muscle in tench : Effects of long-term acclimation to hypoxia. Cell and Tissue Res. 227 : 161-177

Johnston I.A. & Bernard L.M. (1982b). Ultrastructure and metabolism of skeletal muscle fibres in the tench : Effects of long-term acclimation to hypoxia. Cell and Tissue Res. 227 : 179-199

Johnston I.A. & Bernard L.M. (1982c). Utilization of the ethanol pathway by Crucian carp during anoxia. J. exp. Biol. 104 : 73-78

Johnston I.A. & Bernard L.M. (1983). Aquatic and aerial respiration rates, muscle capillary supply and mitochondrial volume density in the air-breathing catfish Clarias mossambicus acclimated to either aerated or hypoxic water. J. exp. Biol. 105 : 317-338

Johnston I.A. & Goldspink G. (1973a). A study of the swimming performance of the Crucian carp, Carassius carassius, L., in relation to the effects of exercise and recovery on biochemical changes in the myotomal muscles and liver. J. Fish Biol. 5 : 249-260

Johnston I.A. & Goldspink G. (1973b). Quantitative studies of muscle glycogen utilization during sustained swimming in Crucian carp, Carassius carassius. J. exp. Biol. 59 : 607-615

Johnston I.A. & Goldspink G. (1973c). A study of glycogen and lactate in the myotomal muscles and liver of the coalfish (Gadus virens, L.) during sustained swimming. J. mar. Biol. Ass. U.K. 53 : 17-26

Johnston I.A. & Maitland B. (1980). Temperature acclimation in Crucian carp : A morphometric analysis of muscle fibre ultrastructure. J. Fish Biol. 17 : 113-125

Johnston I.A. & Moon T.W. (1980a). Endurance exercise training in the fast and slow muscles of a teleost fish (Pollachius virens). J. Comp. Physiol. (B) 135 : 147-156

Johnston I.A. & Moon T.W. (1980b). Exercise training in skeletal muscle of brook trout (Salvelinus fontinalis). J. exp. Biol. 87 : 177-194

Johnston I.A. & Moon T.W. (1981). Fine structure and metabolism of multiply innervated fast muscle fibres in teleost fish. Cell and Tissue Res. 219 : 93-109

Johnston I.A. & Tota B. (1974). Myofibrillar ATPase in the various red and white trunk muscles of the tunny (Thunnus thynnus L.) and the Tub Gurnard (Trigla lucerna L.). J. comp. Biochem. Physiol. 49 : 367-373

Johnston I.A., Davison W. & Goldspink G. (1977). Energy metabolism of carp swimming muscles. J. comp. Physiol. 114 : 203-216

Johnston I.A., Frearson N. & Goldspink G. (1972). Myofibrillar ATPase activities of red and white muscles of marine fish. Experientia, 28 : 713-714

Johnston I.A., Patterson S., Ward P.S. & Goldspink G. (1974). The histochemical demonstration of myofibrillar adenosine triphosphatase activity in fish muscle. Can. J. Zool. 52 : 871-877

Johnston I.A., Ward P.S. & Goldspink G. (1975). Studies on the swimming muscalature of the rainbow trout. I. Fibre types. J. fish Biol. 7 : 451-458

Jonas R.E.E. & Bilinski E. (1964). Utilization of lipids by fish III. Fatty acid oxidation by various tissues from sockeye salmon, (Oncorhynchus nerka). J. Fish Res. Bd. Can. 21 : 653-656

Jones D.R., Randall D.J. & Jarman G.M. (1970). A graphical analyses of oxygen transfer in fish. Resp. Physiol. 10 : 285-298

Kilarski W. (1966). The organisation of the sarcoplasmic reticulum in fish skeletal muscles III. Pike (Esox lucius L.) Bull. Acad. Pol. Sci. (Biol). 8 : 575-579

Kilarski W. (1967). The fine structure of striated muscles in teleosts. Z. Zellforsch 79 : 562-580

Kilarski W. (1973). Cytomorphometry of sarcoplasmic reticulum in the extrinsic eye muscles of the teleost Tinca tinca L. Z. Zellforsh 136 : 535-544

Korneliussen H., Dahl H.A. & Paulsen J.E. (1978). Histochemical definition of muscle fibre types in the trunk muscalature of a teleost fish (the cod, Gadus morhua L.) Histochemistry. 55 : 1-16

Krough A. (1922). 'The Anatomy and Physiology of Capillaries'. New Haven,Yale University Press, pp 276

Kryvi H. (1977). Ultrastructure of the different fibre types in axial muscles of the sharks Etmopterus spinax and Galeus melastomus. Cell and Tissue Res. 184 : 287-300

Kryvi H. & Totland G.K. (1978). Fibre types in locomotory muscles of the cartilaginous fish Chimaera monstrosa. J. fish. Biol. 12 : 257-265

Kryvi H., Flood P. & Guljaev D. (1980). The ultrastructure and vascular supply of the different fibre types in the axial muscles of the sturgeon, Acipenser stellatus. Cell and Tissue Res. 212 : 117-126

Kutty M.N. (1968a). Respiratory quotients in goldfish and rainbow trout. J.Fish. Res. Bd. Can. 25 : 1698-1728

Kutty M.N. (1968b). Influence of ambient oxygen on the swimming performance of goldfish and rainbow trout. Can. J. Zool. 46 : 647-653

Kutty M.N. (1972). Respiratory quotient and ammonia excretion in Tilapia mossambica. Mar. Biol. 16 : 126-133

Le Peuch C.J., Demaille J. & Pechere J.F. (1978). Radioelectrophoresis : a specific microassay for parvalbumins, application to muscle biopsies from man and other vertebrates. Biochem. Biophys. Acta. 537 : 153-159

Lomholt J.P. & Johansen K. (1978). Hypoxia acclimation in carp - how it affects O_2 uptake, ventilation and O_2 extraction from water. Physiol. Zool. 52 : 38-49

Lorenzini S. (1678). "Observazioni intorno alle Torpedeni". Onofri.

Love R.M. (1970). "The Chemical Biology of fishes". London and New York Academic Press.

Lowey S. & Risby D. (1971). Light chains from fast and slow muscle myosins. Nature, Lond. 234 : 81-85

McArdle H.J. & Johnston I.A. (1981). Ca^{2+} uptake by tissue sections and biochemical characteristics of sarcoplasmic reticulum isolated from fish fast and slow muscles. Eur. J. Cell Biol. 25 : 103-107

Magnuson J.J. (1973). A comparative study of adaptations for continuous swimming and hydrostatic equilibrium of scombroid and xiphoid fishes. U.S. Fish. Wildl. Serv. Bull. 71 : 337-356

Marvin D. & Burton D. (1973). Cardiac and respiratory response of rainbow trout, bluegill and brown bullhead catfish during rapid hypoxia and recovery under normoxic conditions. Comp. Biochem. Physiol. 46A : 755-765

Marvin D. & Heath A.G. (1968). Cardiac and respiratory responses to hypoxia in three species of fish. Comp. Biochem. Physiol. 27 : 349-355

Mathieu O., Cruz-Orive L.M., Hoppeler H., Weibel E.R. (1980). Measuring error and sampling variation in stereology : Comparison of various methods of planar image analysis. J. Microscopy. 131 : 75-88

Mathieu O., Krauer R., Hoppeler H., Gehr P., Lindstedt S.L., Alexander R. McN., Taylor C.R. & Weibel E.R. (1981). Design of the mammalian respiratory system. VII. Scaling mitochondrial volume in skeletal muscle to bodymass. Resp. Physiol. 44 : 113-128

Mathur G.B. (1967). Anaerobic respiration in a cyprinoid fish, Rasbora daniconius (Ham). Nature Lond. 214 : 318-319

Matsuvra F. & Hashimoto K. (1954). Chemical studies of the red muscle ("chai") of goldfishes. II. Determinations of the contents of haemoglobin, myoglobin and cytochrome C in the muscles of fishes. Bull. Jpn. Soc. Scient. Fish. 20 : 308-312

Miller A.T. & Hale D.M. (1970). Increased vascularity of brain, heart and skeletal muscles of polycythemic rats. Am. J. Physiol. 219 : 702-704

Moon T.W. & Johnston I.A. (1980). Starvation and the activities of glycolytic and gluconeogenic enzymes in skeletal muscles and liver of the plaice, (Pleuronectes platessa. J. Comp. Physiol. 136 : 31-38

Morgan T.E., Cobb L.A., Short F.A., Ross R. & Gun D.R. (1971). Effects of long-term exercise on human muscle mitochondria. "Muscle Metabolism during Exercise", ed. B. Pernow & B. Saltin 87-95. New York : Plenum. 558 pp.

Mosse P.R.L. (1978). The distribution of capillaries in the somatic muscalature of two vertebrate types with particular reference to teleost fish. Cell and Tissue Res. 187 : 281-303

Mosse P.R.L. (1979). Capillary distribution and metabolic histochemistry of the lateral propulsive muscalature of pelagic teleost fish. Cell and Tissue Res. 203 : 141-160

Moussa T.A. (1957). Physiology of the accessory respiratory organs of the teleost Clarias lazera (C & V). J. Exp. Zool. 136 : 419-454

Muller W. (1976). Subsarcolemmal mitochondria and capillarisation of soleus muscle fibres in young rats subjected to endurance training. Cell and Tissue Res. 174 : 367-389

Nag A.C. (1972). Ultrastructure and adenosine triphosphatase activity of red and white muscle fibres of the caudal region of a fish, Salmo gairdneri. J. Cell. Biol. 55 : 42-57

Naik N.T. (1963). Technical variations of Koelle's histochemical method for demonstrating cholinesterase activity. Quart. J. Micro. Sci. 104 : 89-100

Nishihara H. (1967). Studies on the fine structure of red and white fin muscles of the goldfish, Carassius carassius. Archvm. histol. Jap. 28 : 425-447

Nursall J.R. (1956). The lateral muscalature and the swimming of fish. Proc. Zool. Soc. Lond. 126 : 127-143

Page S.G. (1968a). The structure of tortoise skeletal muscle. J. Physiol. 197 : 709-715

Page S.G. (1968b). Structure of the sarcoplasmic reticulum in vertebrate muscle. Br. Med. Bull. 24 : 170-173

Patterson S. & Goldspink G. (1972). The fine structure of red and white myotomal muscle fibres of the coalfish (Gadus virens). Z. Zellforsch mikrosk. Anat. 133 : 463-474

Patterson S. & Goldspink G. (1973). The effect of starvation on the ultrastructure of the red and white myotomal muscles of the Crucian carp (Carassius carassius). Z. Zellforsch. Mikrosk. Anat. 146 : 375-384

Pearse A.G.E. (1960). In 'Histochemistry' 2nd ed. Churchill, Livingstone, Edinbugh and London.

Pechere J.F., Derancourt J. & Harech J. (1977). The participation of parvalbumins in the activation-relaxation cycle of vertebrate fast skeletal muscle. F.E.B.S. Lett. 75 : 111-114

Penney R., Johnston I.A. & Goldspink G. (1979). Temperature adaptation of fish muscle. "1st International Congress of Physiology and Biochemistry", Vol. 2. Oxford : Pergamon, in press

Perry S.V. (1979). The regulation of contractile activity in muscle. Trans. Biochem. Soc. 7 : 593-617

Pette D., Wimmer M. & Nemeth P. (1980). Do enzyme activities vary along muscle fibres? Histochemistry 67 : 225-231

Pritchard A.W., Hunter J.R. and Lasker R. (1971). The relation between exercise and biochemical changes in red and white muscles and liver in the jack mackerel, Trachurus symmetricus. Fish. Bull. U.S. Fish. Wildl. Serv. 69 :379-386

Prosser C.L. (1973). Oxygen : Respiration and metabolism. In 'Comparative Animal Physiology' Ed. C.L. Prosser. W.B. Saunders & Co. pp 165-206

Prosser C.L., Barr L.M., Pinc R.A. & Lauer C.V. (1957). Acclimation to reduced oxygen in goldfish. Physiol. Zool. 30 : 137-141

Rayner M.D. & Keenan M.J. (1967). Role of red and white muscles in the swimming of the skipjack tuna. Nature Lond. 214 : 392-393

Reid G.K. (1961). "Ecology of Inland Waters and Estuaries". New York Reinhold.

Salamonski J.H. & Johnston I.A. (1982). Capillary supply and mitochondrial volume density in the axial muscles of a mesopelagic teleost, Argyropelecus hemigymnus. Mar. Biol. 69 : 1-5

Saulbert C.W., Armstrong R.B., Shepherd R.E. & Gollnick P.D. (1973). Anaerobic enzyme adaptations to sprint training in rats. Pflugers Arch. 341 : 305-312

Saunders R.L. (1962). The irrigation of the gills in fish. II. Efficiency of oxygen uptake in relation to respiratory activity and concentrations of oxygen and carbon dioxide. Can. J. Zool. 40 : 817-862

Sharp G.D. & Pirages S. (1978). The distribution of red and white swimming muscles, their biochemistry and the biochemical phylogeny of selected scombrid fishes. In 'The physiological ecology of tunas' : 41-78. Eds. G.D. Sharp & A.E. Dizon. New York & London : Academic Press.

Shoubridge E.A. & Hochachka P.W. (1981). Ethanol : Novel end-product of vertebrate anaerobic metabolism. Science. 209 : 308-309

Sing B.N. & Hughes G.M. (1971). Respiration of an air breathing catfish, Clarias batrachus L. J. Exp. Biol. 55 : 421-434

Smit H., Amelink-Koutstall J.M., Vigverberg J. & von Vaupel-Klein J.C. (1971). Oxygen consumption and efficiency of swimming goldfish. Comp. Biochem. Physiol. A30 : 1-20

Smith M.J. & Heath A.G. (1980). Responses to acute anoxia and prolonged hypoxia by rainbow trout (Salmo gairdneri) and mirror carp (Cyprinnus carpio) red and white muscle : use of conventional modified metabolic pathways. Comp. Biochem. Physiol. 66B : 267-272

Spoor W.A. (1946). A quantitative study of the relationship between the activity and oxygen consumption of the goldfish and its application to the measurement of respiratory metabolism in fishes. Biol. Bull. 91 : 312-325

Stanfield P.R. (1972). Electrical properties of white and red muscle fibres of the elasmobranch fish, Scyliorhinus canicula. J. Physiol. Lond. 222 : 161-186

Stevens E.D. & Black E.C. (1966). The effects of intermittent exercise on carbohydrate metabolism in rainbow trout, Salmo gairdneri. J. Fish. Res. Bd. Can. 23 : 471-495

Swift J.D. & Lloyd R. (1974). Changes in urine flow rate and haematocrit values of rainbow trout exposed to hypoxia. J. Fish Biol. 6 : 379-387

Tarr H.L.A. (1972). Enzymes and systems of intermediary metabolism. In 'Fish Nutrition'. Ed. J.E. Halver pp 255-326. Academic Press. New York.

Thillart G. van den. (1977). Influence of oxygen availability on the energy metabolism of goldfish, Carassius auratus L. Ph D thesis, State University of Leiden, The Netherlands.

Thillart G. van den. (1982). Adaptations of fish energy metabolism to hypoxia and anoxia. Mol. Physiol. 2 : 49-61

Thillart G. van den., Kesbeke F. & Waarde A. van. (1976). Influence of oxygen on the energy metabolism of goldfish, Carassius auratus L. Comp. Biochem. Physiol. 554 : 329-336

Totland G.K. (1976). Three muscle fibre types in the axial muscles of the Axolotyl (Ambystoma americanum). A quantitative light and electron microscopic study. Cell and Tissue Res. 168 : 65-78

Totland G.K., Kryvi H., Bone Q. and Flood P.R. (1981). Vascularisation of the lateral muscles of some elasmobranchiomorph fishes. J. Fish Biol. 18 : 223-234

Twelves E.L. (1972). Blood volume of two Antarctic fishes. British Antarctic Survey Bulletin. 31 : 85-92

Valdivia E. (1958). Total capillary bed in striated muscle of guinea pigs native to the Peruvian mountains. Am. J. Physiol. 194 : 585-589

Walesby N.J. & Johnston I.A. (1979). Activities of some enzymes of energy metabolism in the fast and slow muscles of an Antarctic teleost fish (Notothenia rossii). Biochem. Soc. Trans. 7 : 659-661

Walesby N.J. & Johnston I.A. (1980). Fibre types in the locomotory muscles of an Antarctic teleost, Notothenia rossii : A histochemical, ultrastructural and biochemical study. Cell and Tissue Res. 208 : 143-164

Walesby N.J., Nicol C.J.M. & Johnston I.A. (1980). Metabolic differentiation of the skeletal muscles of a haemoglobin-less (Champsocephalus gunnari) and red blooded (Notothenia rossii) Antarctic fish. Bull. of the British Antarctic Survey 50.

Wardle C.S. (1978). Non-release of lactic acid from anaerobic swimming muscle of plaice, Pleuronectes platessa, a stress reaction. J. Exp. Biol. 77 : 141-155

Watters K.W. Jnr & Smith L.S. (1973). Respiratory dynamics of the starry flounder, Platichthys stellatus, in response to low oxygen and high temperature. Mar. Biol. 19 : 133-148

Weber R.E. (1982). Intraspecific adaptation of haemoglobin function in fish to oxygen availability. Comp. Biochem. Physiol. in press

Weber R.E. & Lykkeboe G. (1978). Respiratory adaptations in carp blood. Influence of hypoxia, red cell organic phosphates, divalent cations and CO_2. J. Comp. Physiol. 128 : 127-137

Weber R.E., Lykkeboe G. & Johansen K. (1975). Biochemical aspects of the adaptation of haemoglobin-oxygen affinity of eels to hypoxia. Life Sci. 17 : 1345-1350

Weber R.E., Lykkeboe G. & Johansen K. (1976). Physiological properties of eel haemoglobin : hypoxic acclimation, phosphate effects and multiplicity. J. Exp. Biol. 64 : 75-88

Weber R.E. & Wood S.C. (1979). Effects of erythrocytic nucleoside triphosphates on oxygen equilibria of composite and fractionated haemoglobins from the facultative air-breathing Amazonian catfish, Hypostomus and Pterygolichthys. Comp. Biochem. Physiol. 624 : 279-283

Weber R.E., Wood S.C. & Davis B.J. (1979). Acclimation to hypoxic water in facultative air-breathing fish : blood oxygen affinity and allosteric effectors. Comp. Biochem. Physiol. 62A : 125-129

Weibel E.R. (1980). Stereological methods. Vol. 1. Practical methods for biological morphometry. Academic Press, London.

Weibel E.R., Taylor C.R., Gehr P., Hoppeler H., Mathieu O., Maloiy G.M.O. (1981). Design of the mammalian respiratory system IX Functional and structural limits of oxygen flow. Resp. Physiol. 44 : 151-164

Wheeler A. (1978). A key to the fishes of Northern Europe pp 374. Frederick Warne Ltd.

Wolff J., Goerz R., Bar C. & Guldner T.F.H. (1975). Common morphogenetic aspects of various organotypic microvascular patterns. Microvascular Research 10 : 373-395

Wood S.C. & Johansen K. (1972). Adaptation to hypoxia by increased HbO_2 affinity and decreased red cell ATP concentrations. Nature N.B. 237 : 278-279

Wood S.C. & Johansen K. (1973). Organic phosphate metabolism in nucleated red cells. Neth. J. Sea. Res. 7 : 328-338

Zammit V.A. & Newsholme E.A. (1979). Activities of enzymes of fat and Ketone-body metabolism and fat fuels in teleost and elasmobranch fish. Biochem. J. 184 : 313-322